

CHARACTERIZATION OF THE mRNA CAPPING ENZYME SUBUNIT INTERACTION
IN VACCINIA VIRUS MUTANT Dts36

By

BARON DOUGLAS HOLMES MCFADDEN

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This thesis is dedicated to my parents, Grant McFadden and Alex Lucas.

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Abstract of Thesis Presented to the Graduate School
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Baron Douglas Holmes McFadden

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The vaccinia virus mRNA capping enzyme is a heterodimer encoded by the D1R and D12L genes. In addition to catalyzing the addition of the 7-methylguanosine cap to nascent viral mRNA, the capping enzyme has also been implicated in early viral gene transcription termination and intermediate viral gene transcription initiation. Previous studies have failed to address whether interaction between the D1 and D12 subunits is required for these latter two activities. Prior phenotypic characterization of Dts36, a temperature sensitive mutant with a lesion in the D1R gene product (G705D), revealed dysfunction in methyltransferase, early gene transcription termination and intermediate gene transcription initiation activities. In continuation of these studies, we employed *in vitro* co-immunoprecipitation experiments to compare subunit interaction in the wild type and Dts36 subunits. These experiments revealed that the protein-protein interaction between D1 and D12 in Dts36 is compromised. These studies support the hypothesis that binding between D1 and D12 is required not only for viral mRNA capping but also for early gene transcription termination and intermediate gene transcription initiation.

CHAPTER 1 INTRODUCTION

Poxviruses

The Poxvirus family consists of large, complex, double stranded DNA viruses that replicate in a large range of hosts. *Poxviridae* is split into two major subfamilies: *Chordopoxvirinae* infects species in the phylum chordata which includes a wide variety of mammals as well as humans. *Entomopoxvirinae* infects varying species of insects.

Numerous human pathologies are associated with poxvirus infections and these viruses typically originate from the *Orthopoxvirus*, *Parapoxvirus*, *Yatapoxvirus*, and *Molluscipoxvirus* genera. The most infamous poxvirus, variola virus, is the disease causing agent of smallpox and belongs to the *Orthopoxvirus* genus. In some instances, smallpox (*Variola Major*) has been recorded to have a 35%-40% mortality rate and smallpox cumulatively is responsible for over 300 million deaths in the 20th century alone. Smallpox acquired its namesake because it typically manifested in patients with characteristic maculopapular rash and raised pustular lesions. The resulting lesions would often spread over the entire body and in some cases would cover the patient completely. Smallpox inspired Edward Jenner to propose the first ever vaccine in 1796 (1) and gravity of the disease stimulated the WHO to initiate a successful worldwide vaccination campaign in 1967. Smallpox became the first infectious disease to be completely eradicated from the planet (2). Worldwide smallpox vaccination was discontinued in 1980, just after the disease was declared eradicated, and consequently the general public is no longer cross-protected against other poxviruses. As a result, disease causing poxviruses, including monkeypox and cowpox, are beginning to resurface in humans and the number of known cases is steadily rising. There are

worries that these poxviruses will evolve to jump from human to human more efficiently and result in a pandemic similar to that of smallpox. More worrisome still is that these viruses have an animal reservoir and therefore cannot be eradicated the same way as smallpox (3).

Vaccinia virus is the prototypical member of the poxvirus family and is the most commonly studied. Vaccinia virus is a member of the *Orthopoxvirus* genus and was commonly used to vaccinate against smallpox. Vaccinia shares a high degree of similarity both structurally and genetically to variola virus. However, vaccinia is mostly benign in humans and therefore is an excellent model organism for poxviruses. More generally, vaccinia, like all viruses, must encode sophisticated processes into a limited genomic space which makes it an excellent tool for studying basic cellular and molecular biology. Thorough study of vaccinia virus has also led to more generally applicable discoveries like *in vitro* mRNA capping kits (4) and TOPO cloning (5).

Vaccinia virus, like all poxviruses, is unique from other DNA viruses in one major aspect: replication occurs entirely in the cytoplasm of the host cell, bypassing the nucleus completely. In contrast, most DNA viruses replicate in the nucleus of the cell and hijack cellular transcription and DNA replication proteins. Consequently, vaccinia virus encodes the majority of proteins required for these processes (6). Therefore vaccinia virus infection provides an excellent model for studying complex eukaryotic transcription and DNA replication mechanisms in a more simplistic fashion.

Vaccinia Virus Background

Vaccinia virions are large (360 X 270 X 250 nm) and structurally complex, comprising between 70-90 structural proteins. The virion is brick or pillow shaped and is membrane wrapped. Vaccinia virions are present in multiple forms which are

distinguished by the number of outer membranes found on the virion. The simplest virion form, the mature virion (MV), is wrapped in a single membrane. A model for a typical MV is shown in Figure 1-1. Removing the virion membranes reveals a biconcave core with two proteinaceous structures known as lateral bodies flanking on two sides. Within the core resides the linear, double stranded, 200 kbp DNA genome which is most likely housed inside or complexed with a tubular nucleocapsid structure (6, 7).

Vaccinia virus encodes a large number of genes (around 200). Vaccinia gene names consist of a letter and a number designation which is followed by an L or R. The letter designation relates to a specific fragment of the *HindIII* digest of the vaccinia genome. The number designation is the gene location on that particular *HindIII* fragment listed in escalating order from left to right. Finally, the L or R represents whether the gene transcribes to the left or right, respectively. The protein product of the gene is designated in the same way except that the L or R is dropped from the name.

The vaccinia virus infection cycle is complex and is subject to regulation by a temporal cascade (Figure 1-2) and is briefly summarized in this section. Viral entry into the cell can occur by either membrane fusion or actin-mediated macropinocytosis. The mechanism for entry is dependent on viral strain and cell type. Upon cell entry the virion uncoats, releasing the virion core and lateral bodies into the cell cytoplasm (8).

Viral gene expression is split into three stages: early, intermediate and late. Viral mRNAs from all three stages are polyadenylated at the 3' terminal end and capped at the 5' end. The stages of viral transcription will be discussed in greater detail in a later section.

Upon uncoating, early genes are transcribed in the virion core by packaged early transcription proteins and the resulting transcripts are extruded into the cytoplasm through pores in the core wall. Early genes encode proteins required for immunomodulation, DNA replication and nucleotide synthesis as well as transcription factors for intermediate transcription. Following early gene expression, the virion core undergoes a second uncoating, releasing the viral genome into the cytoplasm. At this point, the viral genome replicates most likely utilizing a rolling hairpin strand displacement mechanism. DNA replication results in the establishment of viral factories which are recognized as areas in the cell devoid of cellular organelles where both viral replication and virion morphogenesis take place (6).

Intermediate genes are expressed after the genome replication begins and are transcribed off of the newly synthesized DNA. Intermediate genes encode transcription factors that drive late gene expression. Virion morphogenesis and assembly proteins are expressed late and result in crescent formation in viral factories. Viral crescents are so named because of their appearance in microscopy. More accurately, a crescent is an incomplete spherical concavity made up of a honeycomb lattice of D13 proteins that acts as a scaffold for membrane recruitment. The crescents close up into complete spherical bodies, forming the immature virions (IV). Along with the DNA, the entire early transcription apparatus is packaged into the virion. A series of catalytic cleavages of virion proteins results in the conversion of IV to mature virion (MV). MVs are the most abundant virion form. MVs can convert to wrapped virions (WV) by acquiring two additional trans-Golgi membranes. Both MVs and WVs require cell lysis in order to escape the cell. WVs travel via cellular microtubules to the cell membrane where they

exit the cell by membrane fusion, leaving the outermost envelope behind. These virions are referred to as extracellular virions (EV) which are basically MVs with an additional membrane. EVs typically mediate spread of infection within the host while MVs are suspected to be responsible for spread of the virus from host to host. Vaccinia's ability to infect multiple species and cell types is likely a direct result of the ability to produce multiple virion forms (6, 8).

Viral Transcription

Early Gene Transcription

Transcription of vaccinia genes is regulated in a temporal cascade. Early transcription occurs in the virion core following the first uncoating event (6). Early genes are transcribed by the packaged early transcription proteins and the resulting mRNA is extruded through small pores in the core wall into the cytoplasm of the infected cell (9, 10).

Early genes are controlled by a specific promoter sequence found 12-29 nucleotides upstream of the transcription start site. The promoter sequence varies but follows the consensus $(A/T)_8G(A/T)_8N_{12}(G/A)$ (11). A heterodimer of vaccinia gene products, D6 and A7, known as the virus early transcription factor (VETF) binds to both the early promoter sequence and residues 7-10 downstream of the transcription start site (12, 13). VETF recruits the RNA polymerase to the early promoter via the RNA polymerase associated protein (RAP94). The viral RNA polymerase exists in two forms: an early form and a post-replicative form. The post-replicative form is an eight subunit complex which transcribes intermediate and late genes. The early form is a nine subunit complex made up of the eight subunit complex and an additional subunit (RAP94). This form of RNA polymerase only transcribes early genes. Therefore, RAP94, the early

RNA polymerase subunit, binds to VETF tethering the RNA polymerase to the early promoter (14-16).

The mechanism for early gene transcription initiation is not completely understood. However, it is known that once VETF is bound to the early promoter, it prevents promoter escape by steric hindrance. Evidence suggests that ATP hydrolysis is required to remove VETF from the early promoter site allowing the RNA polymerase to escape the promoter and continue transcription (17-19).

Early transcription elongation requires a viral nucleoside triphosphate phosphohydrolase-I (NPH-I) as well as ATP. In the absence of NPH-I or in conditions of limited nucleotides, early transcription will stall (20, 21). Intermediate and late transcription elongation also shares these requirements. However, they are subject to a larger degree of regulation and will be discussed in a later section.

Early gene transcripts terminate heterogeneously around 30-50 nucleotides downstream of a specific signal sequence TTTTNT, encoded on the non-template strand (22). The termination signal is sensed in the form of UUUUUNU on the RNA strand by the vaccinia mRNA capping enzyme, a heterodimer complex of D1 and D12 proteins. Evidence suggests that the capping enzyme, in response to the signal sequence, stalls the elongation complex. Once stalled, the NPH I, which is bound to the RAP94 subunit, hydrolyzes ATP and dissociates the ternary complex consisting of RNA polymerase, RNA and DNA thereby releasing the transcript (21, 23-26).

Post-Replicative Gene Transcription

Post-replicative gene expression is split into two temporal categories: intermediate and late. Post-replicative transcription differs from early transcription in several ways. Intermediate and late transcripts possess a heterogeneous 5' "poly A

head" sequence due to slippage of the RNA polymerase on the AAA residues (27, 28). The 5' poly A head sequences are not found in early transcripts and their function is unknown, however the poly A head sequences are still 5' capped in the same manner as early transcripts. Post-replicative transcripts are also not terminated at defined signal sequences in the same fashion as early genes are. The early termination signal U5NU is found in post-replicative genes however it is not recognized and is bypassed. Rather, post-replicative genes terminate heterogeneously resulting in 3' ends which vary largely in size. Post-replicative gene expression is also more regulated than early gene expression and includes regulation by host factors (6).

Intermediate gene transcription is controlled by a variable bipartite promoter sequence ((A/T)₈N₁₂TAAA(T/A)GG) which is distinctive from either early or late gene promoters. Multiple factors, both viral and host, regulate intermediate transcription. VITF (Viral intermediate transcription factor) 1, 2 and 3 as well as the viral mRNA capping enzyme are required for intermediate gene transcription *in vitro* (29). The viral mRNA capping enzyme has also been shown to be required for intermediate transcription initiation *in vivo* (30). VITF-1, encoded by vaccinia E4L, is a multifunctional protein that is both an RNA polymerase subunit and an intermediate transcription factor in its monomeric form (31). VITF-3 is a heterodimer of vaccinia early gene products A8L and A23R. VITF-2 is a heterodimer of host proteins, G3BP & p137, which were shown to co-purify with VITF-2 activity *in vitro* (32). There are other factors which are non-essential for *in vitro* transcription but have been shown to influence intermediate gene expression. Viral B1 protein kinase stimulates intermediate transcription *in vivo*. The target for phosphorylation with regards to intermediate gene expression is unknown. However B1

kinase has been shown to phosphorylate vaccinia H5 protein, making H5 a likely candidate for transcription stimulation (33). A host nuclear transcription factor YingYang1 (YY1) is a negative regulator of both intermediate and late gene transcription. YY1 represses transcription by binding the promoter initiator element TAAA(T/A)GG and blocking the RNA polymerase (34, 35).

Late gene transcription is controlled by a late promoter of the consensus sequence (A/T)₆N₉TAAAT (36). Along with the RNA polymerase, three vaccinia proteins, G8, A1 and A2, were shown to be required for transcription *in vitro*. The mechanism for activity of these three late transcription factors is unknown. However, G8 shows a high structural similarity to eukaryotic PCNA (proliferating cell nuclear antigen) proteins which points to the possibility that G8 may act as a sliding clamp recruiting other late transcription factors (37). H5, a multifunctional viral protein, has a stimulatory role in late gene transcription *in vitro* (38). It has been proposed that H5 acts like a hub protein linking numerous viral activities and may be involved in recruiting transcription factors which would explain its roles in both intermediate and late gene transcription (39). Late gene transcription is also regulated by host heterogeneous nuclear ribonucleoproteins (hnRNP), A2/B1 and RBM3, which were shown to co-purify with late transcription activity (40, 41). Not much is known about the function of these transcription factors however multiple binding interactions have been reported. *In vitro* yeast two hybrid experiments have revealed that G8 and A1 proteins interact with each other (42). A1L and H5R gene products have been shown to self-interact *in vitro*. A2 binds to both the G8 and H5 proteins as well as host proteins hnRNPs A2/B1 and RBM3 *in vitro* (43).

Post-replicative transcription terminates heterogeneously resulting in mRNAs that vary greatly in length. Four viral proteins, A18, J3, G2 and H5, are known to play a role in post-replicative 3' end formation. A18 mutant viruses have been shown to direct synthesis of longer-than-normal post-replicative transcripts (44). A18 has been shown to contain both DNA-dependent ATPase and DNA helicase activities and is required for RNA release from the elongation complex *in vitro*. Therefore A18 is a late transcription termination factor (45-47). Phenotypic characterization of G2 and A18 double mutant viruses revealed that mutations in G2 could counteract the phenotype seen in A18 mutants. More specifically, this virus would synthesize normal length post-replicative mRNAs. G2 mutant viruses have been shown to synthesize shorter than normal transcripts. This observation along with the experiments done with the G2 and A18 double mutant virus indicates that G2 is a positive transcription elongation factor for post-replicative genes (48, 49). J3 is also a positive transcription elongation factor and when mutated displays a phenotype almost identical to G2 mutants (50, 51). H5 has been identified as a positive elongation factor for post-replicative genes as well (52). Additionally, H5 has been shown to have endoribonuclease activity and is likely responsible for post-replicative transcript cleavage (39, 53). H5 and G2 have been shown to interact *in vivo* and *in vitro*. H5 also interacts with A18 and the B1 kinase (42, 54). These results indicate a possible mechanism for post-replicative elongation and termination. G2, H5, J3 and A18 are most likely complexed with the RNA polymerase in a ternary elongation complex. In response to unknown signals, transcription elongation will slow or stall resulting in ternary complex dissociation and transcript release.

Messenger RNA Capping

Messenger RNA Cap Function

Eukaryotic and viral messenger RNAs are co-transcriptionally modified with the addition of a 7-methylguanosine cap to the 5' end of the initiating nucleoside.

Messenger RNA capping is necessary for the following reasons: the cap stabilizes the mRNA and protects it against degradation from cellular exoribonucleases (55); it is recognized by splicing machinery and is therefore required for pre-mRNA splicing and nuclear export (56-58); finally, the cap mediates targeting of the mRNA to ribosomes and is vital for efficient translation. Cellular eIF4E (Eukaryotic translation initiation factor 4E) complex recognizes and binds the mRNA cap and promotes translation.

Structurally, the methylation of the guanosine appears to be important for translation efficiency and the guanosine residue itself appears to confer stability to the transcript (59-61). Additionally, the initiating nucleoside is also frequently methylated. This methylation has been shown to be important for recognizing host versus foreign RNA transcripts. Therefore, a non-methylated initiating nucleoside can result in an innate immune response (62).

Messenger RNA Cap Structure

mRNA cap structure varies depending on the organism and the context. Lower eukaryotes typically possess type 0 cap structures (Figure 1-3). The type 0 cap is the most simplistic structure with guanosine attached to the initiating nucleoside via a 5'-5' triphosphate bridge with a single methylation at the 7th nitrogen on the guanosine. All cap structure types have a 7-methylguanosine group however the other cap types are additionally modified.

Higher eukaryotes typically have a type 1 or 2 cap structure (Figure 1-3). Type 1 caps are additionally methylated at the second oxygen of the ribose in the initiating nucleoside. Type 2 caps are further methylated on the ribose 2-oxygen on the second nucleoside residue. Type 0 and type 1 cap reactions are catalyzed in the cell nucleus of eukaryotic cells. In contrast, the methylation of the second nucleoside in type 2 caps is catalyzed in the cell cytoplasm.

Other variants of the cap include a type 4 cap which involves methylation of the first four nucleosides as well as hyper-methylation of the initiating nucleoside. TMG (2,2,7 trimethylguanosine) cap structures are present in eukaryotic snRNAs (small nuclear RNAs) and have been identified in a few non-eukaryotic organisms. (63, 64).

Messenger RNA Cap Synthesis

Cap 1 structure is the most abundant type in eukaryotes and is the cap structure that is found in vaccinia virus mRNA. There are two known mechanisms for mRNA cap synthesis, type 1 and type 2. The type 2 mechanism is an uncommon pathway that has been shown in vesicular stomatitis virus and is likely utilized by other negative strand RNA viruses as well. Eukaryotic cells and most other viruses, including vaccinia virus, utilize the type 1 mechanism. Therefore only the type 1 mechanism for synthesis cap 1 structures will be outlined here.

Messenger RNA capping is the result of a series of four enzymatic reactions (Figure 1-3). The enzymatic reactions are as follows: 1) An RNA triphosphatase hydrolyzes the γ phosphate from the initiating nucleoside of nascent RNA; 2) A guanylyltransferase removes a pyrophosphate off of a GTP molecule and transfers the resulting GMP via an enzyme-GMP intermediate onto the diphosphate end of the RNA. Reactions 1 and 2 are reversible and result in a guanosine attached to the 5' nucleoside

of the nascent RNA via a 5'-5' triphosphate bridge; 3) Guanine-N7 methyltransferase irreversibly transfers a methyl group from an S-adenosylmethionine (SAM) donor molecule to the N7 atom of the guanine cap. These first three enzymatic reactions result in a 7-methylguanosine or type 0 cap; 4) The ribose 2' hydroxyl group on the initiating nucleoside is methylated by a 2'O-ribose methyltransferase resulting in a type 1 cap structure (63, 64).

Vaccinia Virus mRNA Capping Enzyme

Vaccinia virus replicates entirely in the cytoplasm of the host cell and consequently is unable to hijack nuclear enzymes to process viral RNA. As a result, vaccinia virus encodes the majority of proteins required for mRNA synthesis including a viral mRNA capping enzyme. Viral mRNA is capped in a fashion that mimics host mRNA and is necessary for many of the reasons stated previously. Namely, the mRNA cap stabilizes the RNA and protects against host cytoplasmic exoribonucleases. The messenger RNA cap is involved in directing the viral transcript to ribosomes. Therefore viral protein expression is reliant on mRNA capping in order to utilize the host's ribosomes and translational machinery (65). Additionally, uncapped mRNA is degraded in cellular granular compartments called P-bodies and initiates an innate antiviral interferon response (66, 67). Therefore, viral mRNA capping is necessary in order to evade host immune detection.

The vaccinia capping enzyme is a heterodimer comprised of the vaccinia D1 and D12 proteins. The large (97 kDa) and small subunit (33 kDa) of the heterodimer are encoded by D1R and D12L respectively. D1R and D12L are both controlled by early/late promoters and are therefore constitutively expressed during the infection. The

capping enzyme is also packaged into the virions in order to cap early gene transcripts in the subsequent infection (68-70).

The vaccinia D1/D12 heterodimer is a multi-functional enzyme that has been shown to be involved in two additional processes aside from mRNA capping: early gene transcription termination and intermediate gene transcription initiation (29, 30, 71, 72). The mRNA capping enzyme activities are discussed in more detail in the following sections.

Vaccinia mRNA Capping

The D1/D12 heterodimer catalyzes the first three capping reactions as described in Figure 1-3 to generate a type 0 cap (73). The last reaction, methylation of the initiating nucleoside, is catalyzed by a separate viral protein (J3) forming the final type 1 cap (74).

The large subunit, D1, possesses the domains for all three enzymatic reactions catalyzed by the capping enzyme. The RNA triphosphatase and guanylyltransferase activities are located in the N terminus of the D1 protein as an autonomous domain. However, the triphosphatase and guanylyltransferase can be mutationally inactivated individually without affecting the other enzymatic activity (75, 76). These reactions do not require the small D12 subunit for activity (77, 78). The (guanine-N7-) methyltransferase domain is located in the C-terminus of D1 and is separated from the triphosphatase and guanylyltransferase domain by a proteolytic hinge region (79). Unlike the triphosphatase and guanylyltransferase activities, the methyltransferase in D1 is dependent on the small subunit, D12. D12 does not contain any intrinsic enzymatic function and it is required only to allosterically activate the methyltransferase activity in the D1 subunit (79, 80).

The RNA triphosphatase is a member of metal-dependent phosphohydrolase protein family (81) and requires magnesium for activity (82). The RNA triphosphatase active site involves the following residues: Lys107, Glu126, Asp159, Lys161, Glu37, Glu39, Arg77, Glu192, and Glu194. These nine amino acids are essential for activity and are completely conserved in all poxviruses (77, 81, 83, 84).

The guanylyltransferase belongs to a covalent nucleotidyl transferase superfamily which consists of five conserved domains with a high degree of similarity regarding amino acids and residue spacing. The guanylyltransferase active residue is K260 which binds GMP covalently. K260 is located in the KxDG motif and is conserved in a wide variety of viral and yeast guanylyltransferases as well as in mammalian, viral and yeast DNA or RNA ligases. Residues gly263, glu375, gly376, lys399, thr398 and asp400 are necessary for enzyme-GMP complex formation *in vitro*. Residues glu375, lys392 and asp400 are important for GTP binding affinity and the glu375 and lys392 residues are important for catalysis (75, 85).

The methyltransferase activity of the capping enzyme is the most thoroughly researched. The methyltransferase domain in D1 has weak intrinsic activity. D12 binds D1 and allosterically activates the D1 methyltransferase, increasing activity 30-100 fold *in vitro* (79, 80). D12 alters D1's conformation bringing SAM into closer proximity with D1's SAM binding residues. Therefore D12 increases D1's affinity for the SAM, which in turn promotes methyltransferase activity (86). D12-like proteins are only present in some poxviruses and there are no known cellular homologs of D12. D1 Protein sequences IHYSF (amino acids 681-685) and VLAIDFGNG (amino acids 594-602) are conserved between Shope fibroma virus, African swine fever virus, and *Saccharomyces*

cerevisae methyltransferases. The Gly-600 in the VLAIDFGNG motif was found to be critical for catalysis (80, 87, 88). Residues Asn550, Tyr555, Phe556, Arg560, Arg562, Asn570, Lys573, Lys607, Tyr608, Asp676, Phe679, His682, and Glu763 in D1 have been shown to be essential for methyltransferase activity in an *in vivo* yeast complementation assay. These D1 mutations have no effect on D1/D12 subunit interaction (89, 90).

In vitro biochemical studies coupled with analysis of the solved crystal structure of the C-terminal domain of D1 have led to the following conclusions: 1) Residues his682 and tyr683 in the IHYSF domain as well as the glu763 and phe679 residues form hydrogen bonds with the mRNA cap guanosine. Phe679 also plays a key role in forming the hydrophobic pocket; 2) Residues asn601, arg632, arg560 and arg562 are involved in non-structural roles with the mRNA cap triphosphate with asn601 and arg632 binding directly to the cap triphosphate; 3) Tyr555, phe556, lys573, asp598, gly600, asp620 and tyr683 are essential for non-structural roles, most likely involving binding and orientation of the methyl donor SAM; 4) Asn550 plays a structural role but is still essential for methyltransferase activity (89-91).

Early Transcription Termination Activity

The capping enzyme was found to co-purify with early gene transcription termination activity biochemically (72). The capping enzyme induces termination in *in vitro* transcription assays and is required for early transcription termination activity *in vivo* (30, 71). Early gene transcription termination requires the U5NU sequence in the nascent RNA (92, 93), the RNA polymerase, RAP94, the capping enzyme, NPH-I and ATP hydrolysis. Several interactions between these elements are already known. The N-terminal domain of D1 has an RNA binding domain which has a higher affinity for U

rich sequences *in vitro* (78). The D1 N-terminal domain has also been shown to crosslink with U5NU *in vitro*. The early RNA polymerase subunit, RAP94, also interacts with the U5NU sequence. The interaction of D1 and RAP94 with termination signal is not mutually exclusive (23). It is also known that the capping enzyme interacts with the RNA polymerase *in vivo* (94). Furthermore, interaction between NPH-I and RAP94 is required for termination activity (95).

The early transcript release can be separated into two steps: a sensing step and a transcript release step. The capping enzyme is only required for the sensing step while NPH-I is necessary for transcript release. Biochemical evidence shows that stalled elongation complexes can trigger transcript release even in the absence of both U5NU and the capping enzyme (23).

Evidence suggests the following mechanism for early termination: the capping enzyme and NPH I are bound to the early RNA polymerase in a ternary elongation complex. When the U5NU signal is extruded from the ternary complex, it is sensed by the capping enzyme which stalls the elongation complex. At this point, NPH-I hydrolyzes ATP and forces transcript release by a forward translocation mechanism (23, 96).

It is important to note that early transcription termination activity of the capping enzyme is independent from the mRNA capping activity. More specifically, mutant D1 proteins deficient in guanylyltransferase or methyltransferase activity are still capable of transcript termination (25). Both subunits of the capping enzyme are required for early termination *in vitro* as shown in two independent studies (24, 25).

Intermediate Transcription Initiation Activity

Very little is known about intermediate transcription initiation. However, the vaccinia capping enzyme has been shown to be essential for intermediate transcription initiation, biochemically and *in vivo* (29, 30). It is suspected that the capping enzyme plays a structural role in the transcription initiation complex (29). Transcription initiation activity has been shown to be independent of mRNA capping. More specifically, mutant D1 that is deficient in transcript guanylylation retains intermediate transcription activity, *in vitro* (97). Both subunits of the RNA capping enzyme are required for intermediate transcription initiation *in vitro* (24). The role that the capping enzyme plays in intermediate transcription initiation has yet to be elucidated.

Dts36: A Mutant in the mRNA Capping Enzyme

Temperature sensitive mutants are one of the most important tools for studying the roles of vaccinia virus proteins. Dts36 is a previously characterized temperature sensitive mutant with a lesion in the large subunit, D1, of the capping enzyme. Dts36 is part of the Dales temperature sensitive mutant collection. Initial phenotypic characterization of Dts36 revealed a lethal sensitivity for viral infection at 40°C with a relative particle forming unit/mL (PFU/mL) ratio between 30°C and 40°C of 3700:1. Dts36 was reported to have a small plaque phenotype regardless of the temperature (98). Dts36 displays a defective early phenotype at the non-permissive temperature, 40°C, which is characterized by a lack of viral DNA replication and post-replicative gene expression. For the purposes of this document the permissive and non-permissive temperatures are 31°C and 40°C respectively. Dts36 also exhibits extended early protein synthesis to late time points post-infection at the non-permissive temperature (30, 99, 100). Genetic characterization of Dts36 indicated that the temperature sensitive

lesion mapped to the D1 gene. Sequencing of the D1 gene revealed a single nucleotide polymorphism changing a glycine to an aspartic acid at residue 705 (G705D) in the methyltransferase domain (30).

Further investigation revealed that Dts36 has inefficient early gene transcription termination activity at the non-permissive temperature. The early gene termination signal, U5NU, is frequently bypassed resulting in longer than normal transcripts that have terminated at downstream U5NU signals. Multiple termination signals are often bypassed before the transcript terminates. This early termination slippage is apparent at the permissive temperature as well but the effect is much more pronounced at the non-permissive temperature. Dts36 is also specifically deficient in intermediate gene expression at the non-permissive temperature. The capping enzyme has previously been shown to be required for intermediate transcription and therefore Dts36 is compromised for initiation activity (30). Preliminary biochemical experiments with Dts36 suggest that the methyltransferase activity is also impaired in Dts36 at both the permissive and non-permissive temperatures *in vitro* (R. Condit unpublished). A compromised methyltransferase activity would result in mRNA transcripts with an unfinished 5' cap. Furthermore, the cap reactions are reversible without the addition of the guanine N-7 methyl group which indicates that the guanosine cap is unstable. In agreement with the previous observation, Dts36 also displays aberrant early mRNA metabolism at the non-permissive temperature. There is a marked decrease in early gene mRNA levels, roughly 1/3 of the levels seen at the permissive temperature (30).

Study Objectives

The underlying cause responsible for the temperature sensitive phenotype in Dts36 has yet to be shown. The mechanism by which Dts36 is temperature sensitive

has yet to be shown. Dts36 is deficient in both early gene transcription termination and intermediate gene transcription initiation *in vivo* (30). Biochemical evidence indicates that Dts36 is deficient in methyltransferase activity *in vitro* as well (Condit unpublished). Studies have shown that methyltransferase activity, early gene transcription termination and intermediate gene transcription initiation all specifically require both the D1 and D12 subunits *in vitro*. Furthermore, both subunits are required for methyltransferase activity in an *in vivo* yeast complementation assay (101). In contrast, the capping enzyme's RNA triphosphatase and guanylyltransferase activities do not require the D12 subunit (77, 78). These observations draw attention to a possible mechanism explaining the thermo-sensitivity of Dts36 infection. A faulty subunit interaction between the mRNA capping enzyme subunits, D1 and D12, would explain all of the deficiencies found in Dts36. Furthermore, this would be the first instance of evidence that subunit interaction is required for early gene transcription termination and intermediate transcription initiation *in vivo*.

In this study, this problem is assessed by examining the D1 and D12 subunit interaction in Dts36. In Chapter 2, evidence is presented indicating that the mRNA capping enzyme subunit interaction is indeed impaired. Furthermore, a subunit interaction deficiency should not affect either the RNA triphosphatase or guanylyltransferase activities. Preliminary data is presented in Chapter 3 suggesting that guanylyltransferase activity of the mRNA capping enzyme is unimpaired.

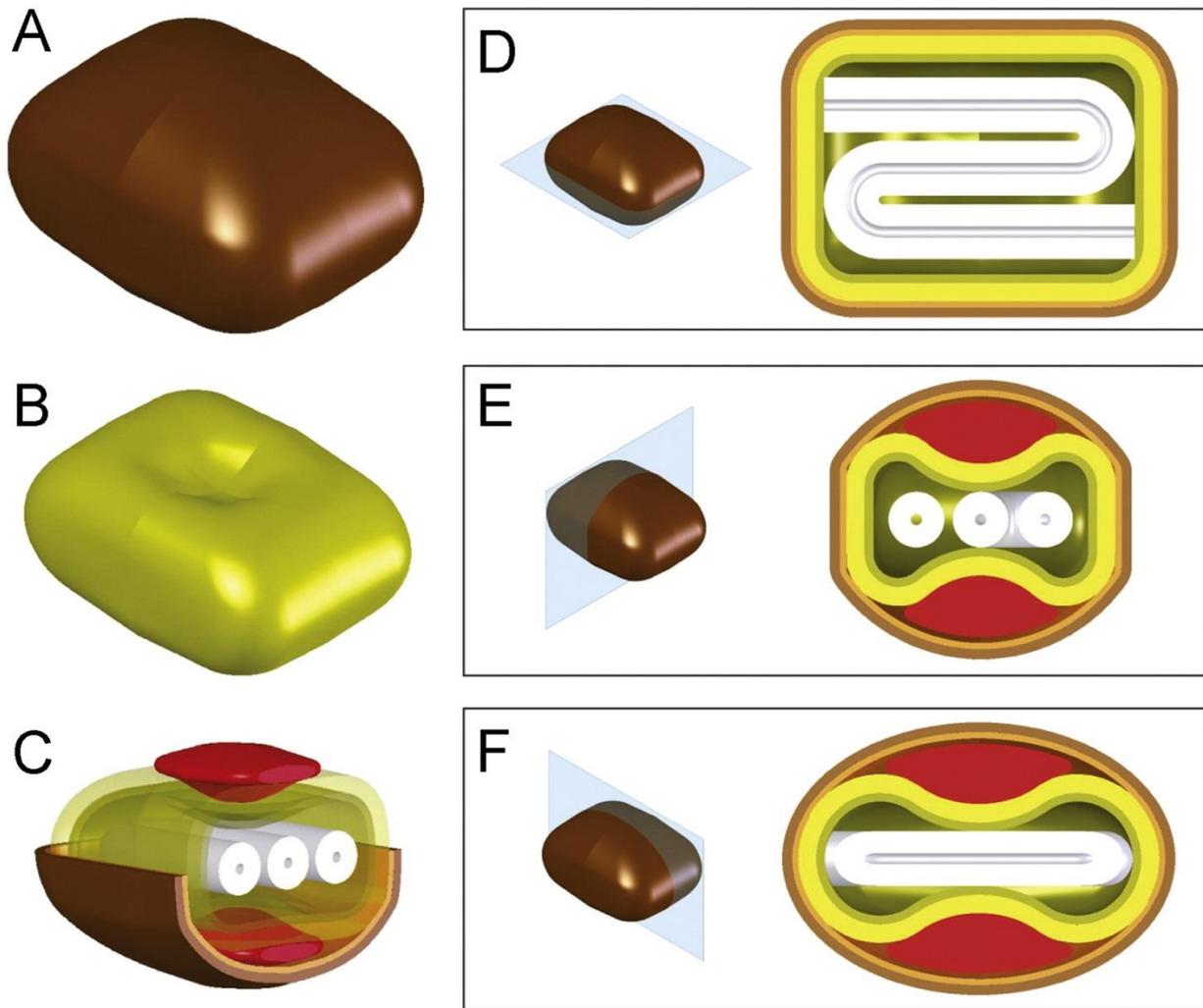


Figure 1-1. Model of vaccinia virus mature virion (MV). (A) The whole virion wrapped in a lipid membrane (brown). (B) Removing the membrane reveals the bi-concave protein core (green). (C) Cross-section of the entire virion showing all layers including the lateral bodies (red) fitting into the core indentations and the nucleocapsid (white) inside of the core. (D-F) Cross-sections of the virion in three mutually perpendicular planes. [Adapted from McFadden, B. D., N. Moussatche, K. Kelley, B. H. Kang, and R. C. Condit. 2012. Vaccinia virions deficient in transcription enzymes lack a nucleocapsid. *Virology*. 434:51 (Fig. 1).]

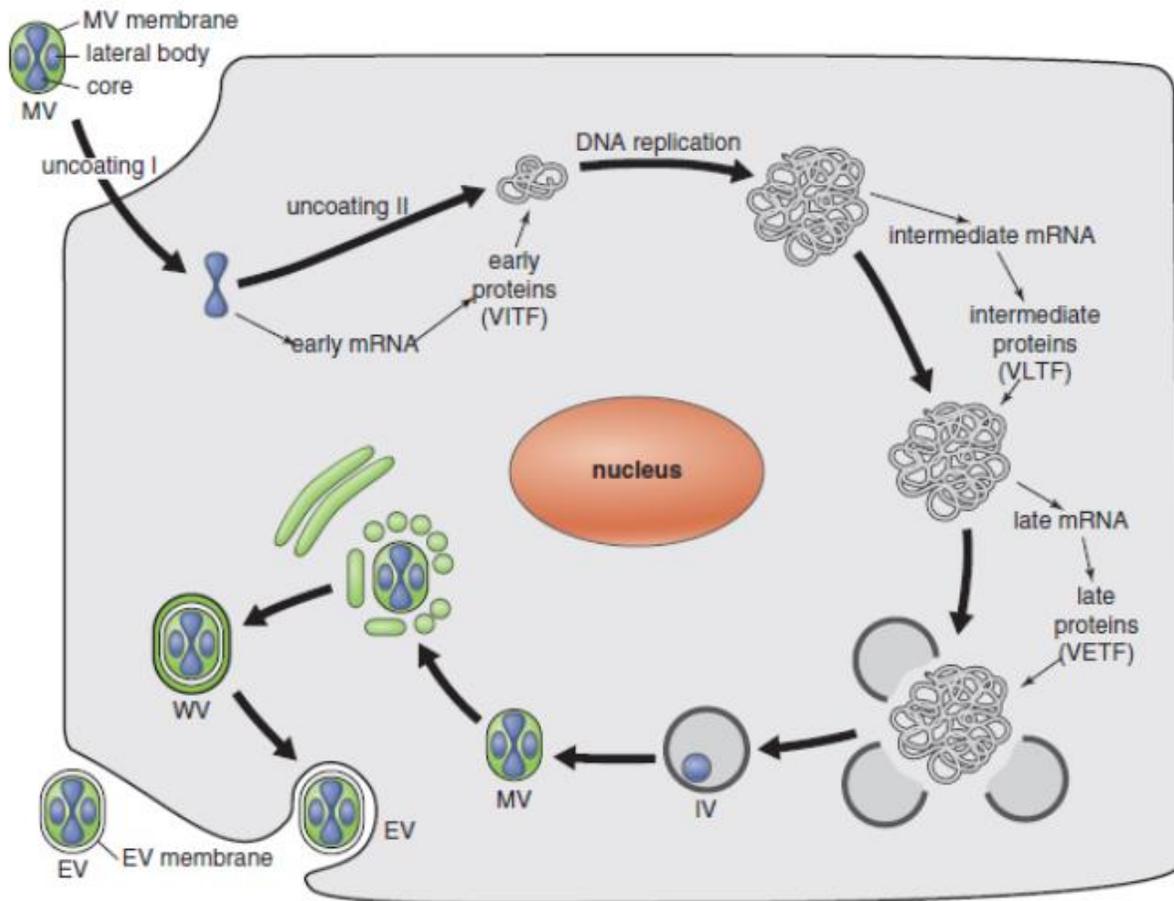


Figure 1-2. Life-cycle of vaccinia virus infection. Virion enters the cell (top left) and discards membrane (uncoating I) extruding the virion core into the cellular cytoplasm. Early gene expression catalyzes temporal cascade leading to DNA replication followed by intermediate and late gene expression. Viral genome and late proteins are packaged into assembled viral crescents. Viral crescents culminate in immature virions (IV) and viral DNA is packaged which is seen as a dense nucleoid (blue circle). Proteolysis converts IVs to mature virions (MV). MVs acquire two additional trans-Golgi membranes and form wrapped virions (WV). Wrapped virions exit the cell by membrane fusion forming extracellular virions (EV). [Adapted from Condit, R., and N. H. Acheson. 2011. Poxviruses, p. 317 (Figure 26.5). In M. Palumbo and L. Morris (eds.), *Fundamentals of Molecular Virology*, 2nd ed. John Wiley & Sons, Inc.]

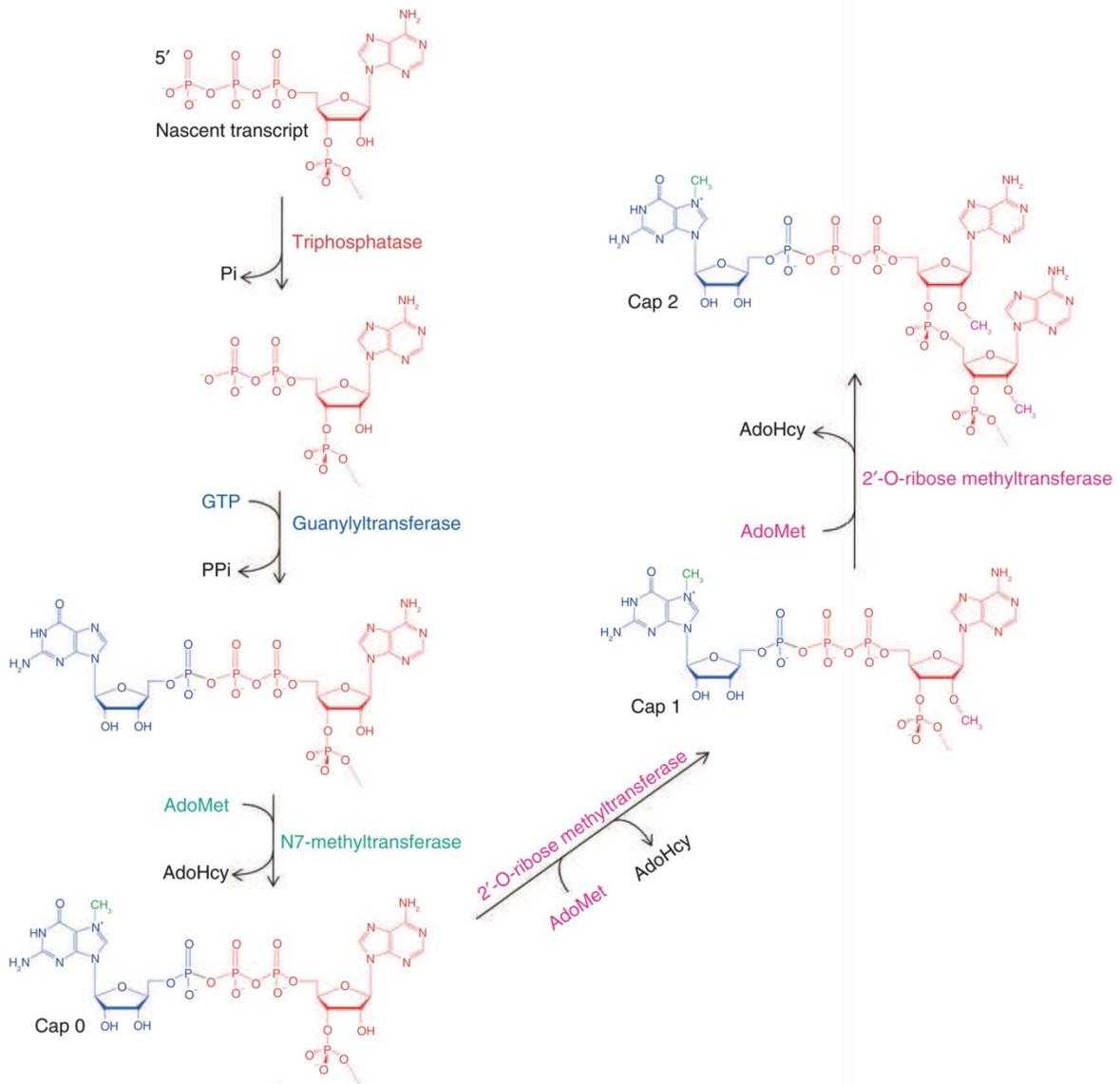


Figure 1-3. mRNA cap structure types and synthetic reactions. [Adapted from Ghosh, A., and C. D. Lima. 2010. *Enzymology of RNA cap synthesis*. Wiley Interdiscip. Rev. RNA. 1:154 (Figure 1).]

CHAPTER 2 MATERIALS AND METHODS

Cells and Viruses

BSC40 cells, a continuous line of African green monkey kidney cells, were grown and maintained as previously described (103, 104). Growth and propagation of the vaccinia virus wild type strain IHD-W and the temperature sensitive mutation Dts36 were performed as previously described (99). The permissive temperature, 31°C, supported growth of both IHD-W and Dts36 while the non-permissive temperature, 40°C, supported growth of IHD-W but inhibited growth of Dts36.

Virus Purification

Viruses were sucrose-gradient purified as previously described (105).

Viral Extract Preparation

Five OD of purified wild type or mutant virus was mixed with 500mM Tris-HCl, pH 8.0, 100 mM dithiothreitol (DTT) and 0.5% Nonidet P40 (NP40) to a final volume of 800 μ L. Mixtures were incubated at room temperature for 10 minutes and then centrifuged in a micro centrifuge for 2 minutes at maximum speed. Pellets were resuspended in 150 μ L of 300 mM Tris-HCl pH 8, 250 mM KCl, 50 mM DTT. 11.25 μ L of sodium deoxycholate (DOC) was added and the sample was incubated on ice for 30 minutes. 16 μ L glycerol was added and the sample was centrifuged in a micro centrifuge at top speed for 5 minutes at 4°C. The supernatant was applied to a 150 μ L diethylaminoethyl-cellulose (DEAE) column and the column was eluted in 60 mM Tris-HCl, pH 8.0, 250 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% triton X-100, 3 mM DTT, 10 mM glycerol. The protein concentrations in the eluted fractions were determined by

Bradford assay. The fractions with the highest protein concentration were combined and stored at -80°C.

Viral DNA Isolation

Bsc40 cells were infected with virus at 31°C and DNA was isolated using a Qiagen DNeasy tissue kit as previously described (99).

PCR Amplification, Cloning and Sequencing

The D1R open reading frame was PCR amplified from both IHD-W and Dts36 purified DNA utilizing custom primers (Sigma). The forward primer included a leader sequence encoding a *SacI* restriction site and a single FLAG tag. The reverse primer included a tail with an *XhoI* site. The D12L open reading frame was PCR amplified from IHD-W purified DNA. The forward primer includes a leader sequence encoding an *NcoI* site followed by a single human influenza hemagglutinin (HA) tag. The reverse primer included a tail sequence with a *BamHI* site. The PCR products were cloned into the pCR 2.1-TOPO vector utilizing a TOPO TA cloning kit as outlined by the manufacturer (Invitrogen). TOPO clones were sequenced with primers both internal and external to the open reading frame. Sequencing was performed by University of Florida ICBR DNA sequencing core. D1 and D12 inserts were removed from the TOPO vector by restriction enzyme digestion. The inserts were subcloned into pTM1 expression vector with T4 DNA Ligase (New England Biolabs). pTM1 is a mammalian expression vector that is controlled by a T7 promoter and contains an ampicillin resistance gene. The fusion plasmids were then transformed into chemically competent TOP10 E-coli by heat shock. Transformed E-coli were grown in ampicillin selection media. The plasmids were purified using QIAGEN Plasmid Mega Kit as outlined by the manufacturer (Qiagen).

***In Vitro* Transcription/Translation**

IHD-W or Dts36 Flag-D1 and IHDW HA-D12 were singly or co-expressed using the TnT T7 Coupled Reticulocyte Lysate System as described by the manufacturer (Promega). One or both of the complementing fusion D1/D12 pTM1 plasmids were mixed with 4 μ L of EasyTag L-[³⁵S]-Methionine (PerkinElmer), 1 μ L RNasin RNase inhibitor (Promega), 0.5 μ L protease inhibitor (1:1000) (Sigma) and the kit reagents: 25 μ L rabbit reticulocyte lysate, 2 μ L TnT reaction buffer, 1 μ L TnT T7 RNA polymerase, 1 μ L 1 mM amino acid mixture minus methionine. The reaction was made up to 50 μ L in water and incubated at 30°C for 90 minutes. Singly translated FLAG-D1 product lysates were mixed with translated HA-D12 product lysates. 4 μ L were removed for diagnostic purposes.

Co-immunoprecipitation Assay

Co-immunoprecipitations were performed using the Dynabeads Co-immunoprecipitation kit as described by the manufacturer (Invitrogen). Monoclonal Anti-HA (Sigma) or Monoclonal Anti-FLAG (sigma) antibodies were conjugated to the magnetic Dynabeads as described by the manufacturer (Invitrogen). Mixed FLAG-D1/HA-D12 lysates from the TnT reactions were diluted to 200 μ L in kit extraction buffer A (EBA): 0.1M NaCl, protease inhibitor (1:100) (Sigma), 110 mM potassium acetate, buffering salts (pH 7.4). Lysates were then mixed with antibody conjugated magnetic beads and gently rotated at 4°C for 30 minutes. Alternately, in some experiments, lysates were rotated at 31°C and 40°C. The following steps were performed at room temperature, 31°C or 40°C. Beads were collected using a magnet and the supernatant was discarded. The beads were washed with 200 μ L of EBA. Beads were collected and washed two more times in the same buffer. Beads were then collected and washed in

Long Wash Buffer (LWB) (0.02% Tween, buffering salts pH 7.4) and rotated for 5 minutes. Beads were collected and then resuspended in 40 μ L Laemmli sample buffer (LSB). The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in an 11% gel alongside the Precision Plus Protein Dual Color Standards marker (Bio-Rad). The gels were stained with Coomassie, destained and dried. The gels were visualized by autoradiography and quantified using a Storm phosphorimager (GE Healthcare) and the ImageQuant software program (GE Healthcare).

***In Vitro* RNA Substrate Synthesis**

DNA template (150bp) containing SP6 promoter site and nonspecific sequence was PCR amplified utilizing custom primers (Sigma). Non-specific 138bp RNA product was transcribed off of the template using the MEGAscript SP6 kit as outlined by the manufacturer (Life Technologies). RNA was purified using the RNeasy kit as outlined by the manufacturer (Qiagen). RNA was eluted in 30 μ L water, separated into aliquots and stored at -80°C. The RNA concentration was determined using a NanoDrop (Thermo Scientific).

Guanylyltransferase Assay

Varying amounts of viral extract were diluted in Reaction Buffer (25 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 1 mM DTT). Samples were mixed with Labeling Buffer (25 μ M GTP, 100 μ M SAM (New England Biolabs), 1.2 μ Ci α ³²P-GTP (PerkinElmer), 10 pMole ends of 138bp RNA substrate). Purified vaccinia virus capping enzyme (2.5 units) (New England Biolabs) was diluted in Reaction Buffer and mixed with Labeling Buffer as well. Reactions were incubated at 31°C or 40°C for 1 hour. Samples were mixed with formamide loading dye and separated on 6% urea-polyacrylamide gels. Gels were fixed

in 10% acetic acid, 10% methanol for 40 minutes. Gels were dried using a vacuum dryer (Bio-Rad). Gels were visualized by autoradiography and quantified using a Storm phosphorimager (GE Healthcare) and the ImageQuant software program (GE Healthcare).

Western Blot Analysis

Samples were separated on 11% SDS-PAGE gel and transferred to a nitrocellulose membrane as previously described (105). The membranes were incubated in TBS-T/5% milk (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.1% Tween-20, and 5% nonfat dry milk) blocking solution at room temperature for one hour. Membranes were incubated in anti-D1 primary antibody diluted 1:2000 in TBS-T/5% milk and rotated overnight at 4°C. Membranes were washed 5 times with TBS-T at room temperature. The membranes were incubated with anti-rabbit Ig-conjugated horseradish peroxidase (Santa Cruz Biotechnology) diluted 1:10000 in TBS-T/5% milk for 1.5 hours at 4°C. The membranes are washed 5 times with TBS-T at room temperature and then exposed in Enhanced chemiluminescence detection kit as outlined in the manual (GE healthcare). D1 antibody was supplied by Dr. Edward Niles.

CHAPTER 3 D1/D12 SUBUNIT INTERACTION

In this chapter, studies were conducted to determine if the Dts36 capping enzyme has a compromised subunit interaction. As stated in the introduction, D1 and D12 binding may be important for early gene transcription termination and intermediate gene initiation activities and has previously been shown to be critical for methyltransferase activity. Dts36 is deficient in all three activities. Therefore the interaction between the mRNA capping enzyme subunits, D1 and D12, in both wild type and Dts36 was investigated. Experimentally, this was explored by conducting *in vitro* co-immunoprecipitation experiments by immunoprecipitating one subunit and measuring the level of the binding partner. The wild type (WT) strain utilized in all experiments was vaccinia strain IHD-W. IHD-W is the viral strain from which all of the Dale's temperature sensitive mutants were generated.

Co-immunoprecipitation Analysis

The D1R open reading frame from both the IHD-W and Dts36 strains were PCR amplified to include an N-terminal Flag tag on the resulting fusion protein. Additionally, the D12L open reading frame from IHD-W was PCR amplified in a similar manner to include an HA tag on the N-terminus of the resulting D12 fusion protein. The fusion Flag-D1 and HA-D12 sequence constructs were cloned into separate pTM1 vectors which put both D1 and D12 expression under the control of a T7 promoter.

The Flag-D1 and HA-D12 constructs were singly expressed in rabbit reticulocyte lysate in the presence of T7 polymerase and radiolabeled ³⁵S-methionine. It is important to note that for protein expression, the TnT lysates were incubated at 30°C, the permissive temperature for Dts36 infection. The resulting radiolabeled HA-D12

reticulocyte lysate was mixed with either IHD-W or Dts36 radiolabeled Flag-D1 lysate. The mixed lysates were incubated in the presence of either anti-Flag or anti-HA antibody conjugated magnetic beads at 4°C.

A representative autoradiogram from the α -Flag (D1) co-immunoprecipitation analysis is shown in Figure 3-1A. The last two lanes show the radiolabeled wild type or Dts36 lysate mixture prior to the immunoprecipitation. The first and second lanes depict the D1 immunoprecipitation results of wild type and Dts36, respectively. There was a pronounced decrease in the amount of the small subunit, D12, co-immunoprecipitated with Dts36 D1 when compared to wild type D1. For a more accurate comparison between wild type and Dts36, the D1 and D12 bands from three separate experiments were quantified using a phosphorimager. Additionally, for each experiment, band intensities were measured at three different exposure time points. The results from each time point were calculated as a D12:D1 ratio. This ratio represents the level of D12 protein signal pulled down per arbitrary unit of D1 signal (D12/D1). The D12:D1 ratios calculated for each time point were averaged and the results for the three experiments are tabulated in Table 3-1. In all three experiments, Dts36 displayed a D12:D1 ratio that was approximately 2-fold lower than wild type. The fold difference was calculated by dividing the wild type ratio by the Dts36 ratio.

Immunoprecipitations of the other capping enzyme subunit, D12, were also performed. These experiments were conducted in the same manner as the D1 immunoprecipitations with the exception that anti-HA antibodies were utilized. A representative autoradiogram of a D12 co-immunoprecipitation results is shown in Figure 3-1B. The last two lanes show the reticulocyte lysate mixtures before

immunoprecipitation. The first two lanes show the results of the anti-HA immunoprecipitation. There was a visible decrease in the level of Dts36 D1 co-immunoprecipitated when compared to wild type D1. Three individual experiments were performed and quantified as stated previously with one difference. A D1:D12 (versus a D12:D1 as used previously) was calculated to determine the level of D1 protein pulled down per D12 (D1/D12). The results of the D12 co-immunoprecipitation experiments reveal a D1:D12 ratio that is 2-3 fold lower in Dts36 when compared to wild type. The D12 immunoprecipitation results corroborate the D1 findings, indicating that the subunit interaction of the mRNA capping enzyme in Dts36 is impaired.

Dts36 is a temperature sensitive mutant that shows noticeable but tolerable defects at the permissive temperature while being lethally sensitive at the non-permissive temperature. We wanted to explore if increasing the temperature post-protein synthesis would further affect subunit interaction. It is possible that an increase in temperature would further alter the D1 protein conformation which could lead to a decrease in subunit association.

Experimentally, this was investigated by co-expressing D1 and D12 at 30°C and then raising the temperature to either 31°C or 40°C for the immunoprecipitation steps. An anti-HA immunoprecipitation experiment of the small subunit, D12, is shown in Figure 3-2. The difference in subunit interaction between wild type and Dts36 in this case is harder to judge visually as more Dts36 D12 subunit was immunoprecipitated than wild type D12 at both temperatures. To clarify the difference between wild type and Dts36 subunit binding, the D1 and D12 bands were quantified by phosphorimager and D1:D12 ratios were calculated for both the 31°C and 40°C conditions. The D1:D12

ratios are shown in Table 3-2. Dts36 subunit binding is impaired at both 31°C and 40°C showing a greater than 2-fold reduction when compared to wild type. The level of Dts36 subunit association relative to wild type is comparable to the previous findings (Figure 3-1B). However, no decrease in subunit association was detected for Dts36 at 40°C versus 31°C. Considering Dts36's phenotype, an increase in subunit dissociation was expected at higher temperatures, however manipulating the temperature post-synthesis appears to have no effect on subunit interaction *in vitro*. Experiments were attempted to judge whether protein synthesis at 40°C versus 31°C affected subunit interaction. However, rabbit reticulocyte lysate does not synthesize proteins efficiently or at all at 40°C (data not shown).

SWISS-MODEL: *In Silico* D1/D12 Interaction Analysis

To further investigate the subunit interaction in Dts36, the structure of the D1-D12 interface was examined *in silico*. We were interested in determining if the Dts36 mutation was responsible for any structural changes in D1 which would translate to interaction impairment between the capping enzyme subunits. The crystal structure for the interaction site of D12 with the C-terminal domain (amino acids 545-844) of D1 was previously solved (91). Utilizing the solved wild type structure, we were able to model the C-terminal domain of Dts36 D1 interacting with D12 in the SWISS-MODEL program (<http://swissmodel.expasy.org/>) (Figure 3-3A). The C-terminal domain of Dts36 D1 is shown in blue with the area of contact with D12 shown in lighter blue. The G705D mutation is labeled and depicted in pink. D12 is colored brown and the area of interaction with D1 is colored lighter brown. The important salt bridge and hydrogen bond interactions between the subunits are depicted in red and green, respectively. G705D is found at one end of beta-sheet #5 which is removed from the D1-D12

interface and is not close in proximity to any of the important interaction residues. The nearest D12 residue to the 705A side-chain is over 8 angstroms away and bonding interactions only typically occur within 5-6 angstroms (Figure 3-3B) (106). Therefore, the Dts36 mutation is not directly interfering with subunit binding. Presumably, the mutation causes a more global conformational change in D1 which is interfering with D1-D12 interaction. However, the Dts36 model overlays perfectly with the wild type crystal structure displaying no notable perturbations. Therefore, the SWISS-MODEL program did not calculate any structural abnormalities for Dts36. However, a closer look at the mutated residue reveals some interesting structural aspects. A zoomed in image of the Dts36 ASP705 residue is found in Figure 3-3B. The 705A side-chain sticks out and comes into very close proximity with D1 protein backbone in the adjacent β -sheet (#4). The three closest notable contacts to the terminal oxygens of ASP705 are outlined in Figure 3-3B. More specifically, a terminal oxygen group on ASP705 comes into very close proximity (2.5 and 2.8 angstroms) with two of the backbone oxygens in β -sheet 4. Additionally, the other ASP705 terminal oxygen comes into close contact (2.9 angstroms) with a backbone oxygen within the same β -sheet (#5). It is likely that these three backbone oxygens result in a strong repulsive force against ASP705's terminal oxygens. It is possible that this repulsive force is pushing the two β -sheets away from each other and/or altering the conformation of β -sheet 5. However this is highly speculative.

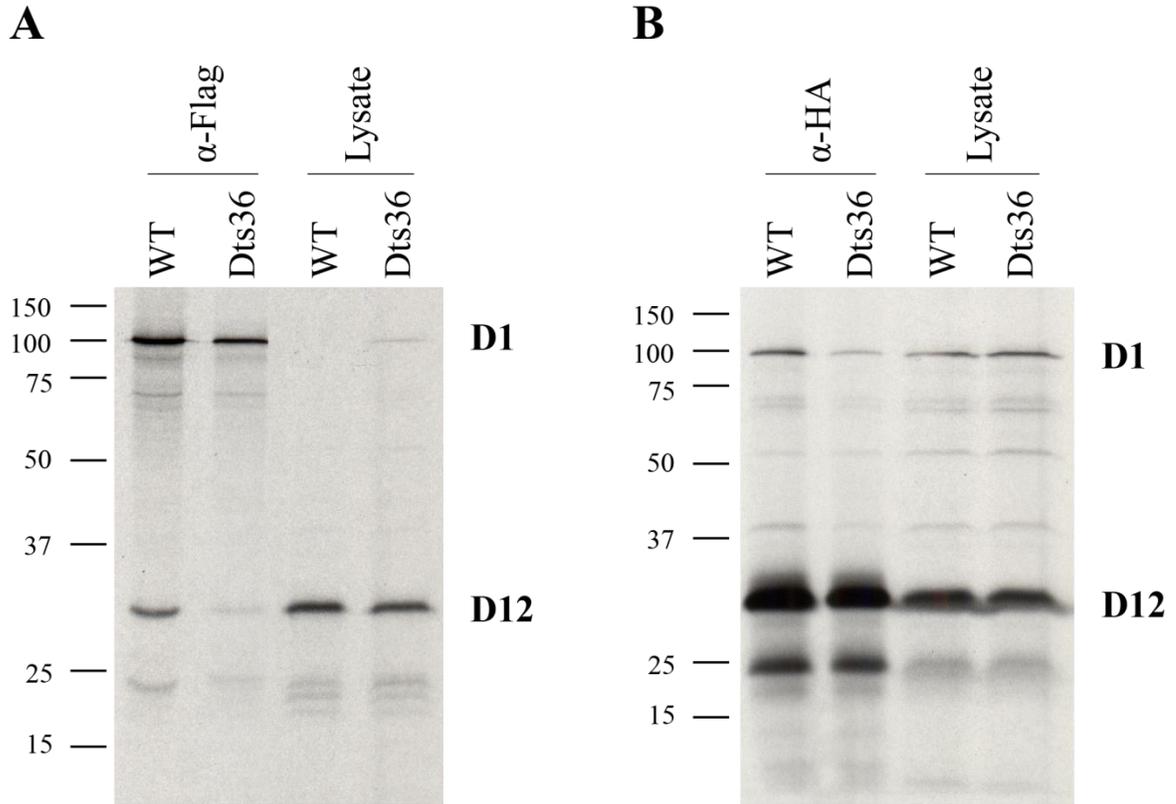


Figure 3-1. Co-immunoprecipitation analysis of D1 and D12 proteins. (A) Representative autoradiogram showing α -Flag (D1) co-immunoprecipitation for WT (IHD-W) and Dts36 in the first two lanes. D1 and D12 rabbit reticulocyte lysate mixtures for WT and Dts36 are shown in the last two lanes. (B) Representative autoradiogram showing α -HA (D12) co-immunoprecipitation for WT and Dts36 in the first two lanes. D1 and D12 rabbit reticulocyte lysate mixtures for WT and Dts36 are shown in the last two lanes. Molecular weight marker indicated on the left and protein designations are indicated on the right for both A and B.

Table 3-1. D1 & D12 co-immunoprecipitation ratios

Sample	α-Flag (D1) Immunoprecipitation			α-HA (D12) Immunoprecipitation		
		Average D12:D1		Average D1:D12		
WT	0.545	0.112	0.148	0.269	0.047	0.022
Dts36	0.215	0.071	0.059	0.171	0.013	0.006
Fold Difference	2.54	1.58	2.53	1.57	3.62	3.61

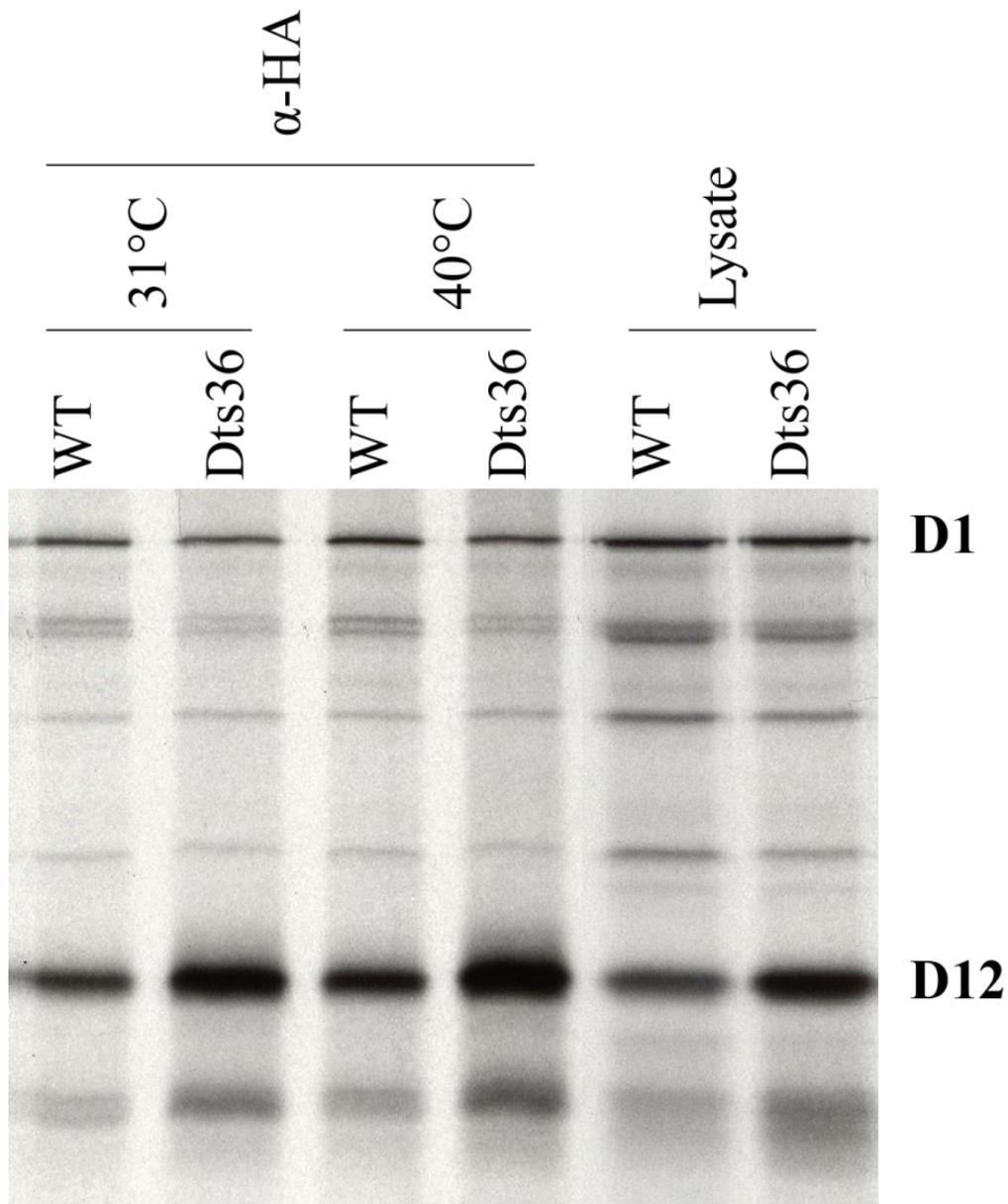


Figure 3-2. D12 co-immunoprecipitation analysis conducted at 31°C or 40°C. A representative autoradiogram showing α -HA (D12) co-immunoprecipitations of WT (IHD-W) in lanes 1 and 3 and Dts36 in lanes 2 and 4. Co-immunoprecipitations conducted at 31°C and 40°C are shown in the first two lanes and lanes 3 and 4, respectively. Co-expressed D1 and D12 rabbit reticulocyte lysates for WT and Dts36 are shown in the last two lanes. Protein designations are indicated on the right.

Table 3-2. D1:D12 ratios for α -HA (D12) co-immunoprecipitations at 31°C or 40°C.

Sample	D12:D1 Ratio:	
	31°C	40°C
WT	0.34	0.36
Dts36	0.14	0.13
Fold Difference	2.47	2.82

Figure 3-3. Model of Dts36 D1 C-terminal domain and D12 interaction site created in SWISS-MODEL. Model is structured off of the solved crystal of WR (wild type strain). (A) Dts36 D1 is colored blue with D12 interaction sites shown in light blue. D12 is colored brown with the D1 interaction site shown in light brown. Dts36 ASP705 mutation is colored pink. Residues involved in salt bridge interactions between D1 and D12 are green. Residues with hydrogen bond interactions between D1 and D12 are red. (B) Zoom-in of the red square area in A displaying the Dts36 mutant residue and notable interactions with ASP705 side-chain. Oxygens are shown in red and nitrogens in blue. The dotted yellow lines indicate notable distance measurements from the ASP705 side-chain (in angstroms).

A



B

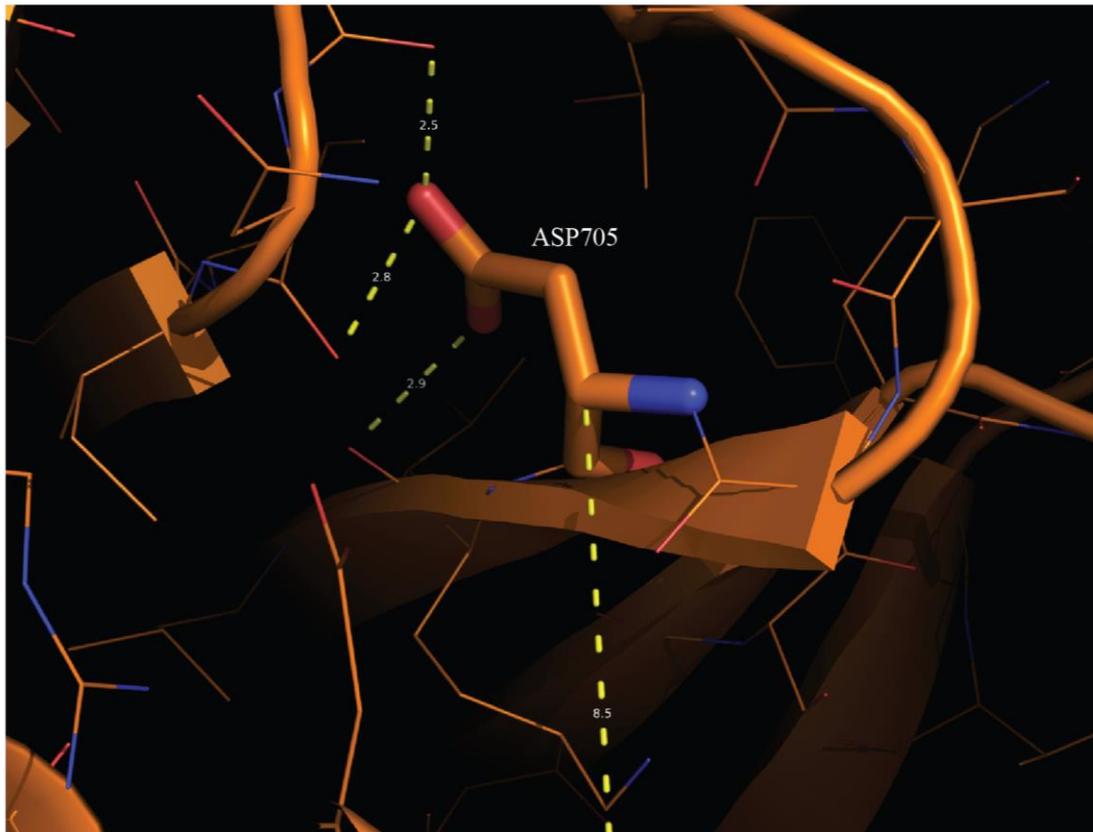


Figure 3-3. Continued

CHAPTER 4 GUANYLYLTRANSFERASE

A faulty capping enzyme subunit interaction would explain the phenotype seen in Dts36. In this scenario, the RNA triphosphatase and guanylyltransferase in D1 should be unimpaired as these activities are not reliant on the D12 subunit. In this chapter, the Dts36 D1 guanylyltransferase activity was investigated to determine if it is deficient. Experimentally, we explored this by performing *in vitro* guanylyltransferase assays with purified virion extracts. Dts36 and wild type (IHD-W) virus were grown under permissive conditions and sucrose-gradient purified. The purified virions were solubilized by treating with NP40 (non-ionic detergent), DTT (reducing agent) and deoxycholate (ionic detergent) to produce viral extracts. Increasing amounts of Dts36 and wild type extracts were mixed with radiolabeled $\alpha^{32}\text{P}$ -GTP, SAM (methyl donor) and an *in vitro* transcribed 138bp RNA product. The reactions were incubated at 31°C or 40°C and then run on a denaturing urea-polyacrylamide gel. Purified vaccinia virus capping enzyme was also incubated under the same conditions minus viral extract as a positive control.

An autoradiograph of the resulting gel is presented in Figure 4-1A. Two radiolabeled bands are present in the gel where there should be only one. There are two possible explanations for this anomaly. The most likely reason is that the RNA substrate was not gel purified and multiple RNA products were transcribed in the original synthesis reaction. The other possibility is RNA degradation as a result of a high concentration of magnesium in the buffer and increased temperatures (107). The issue as of yet is unresolved. Regardless, both bands found in the gel were guanylylated with $\alpha^{32}\text{P}$ -GMP and were therefore analyzed. An increase in band intensity (both bands i and ii) correlated with increasing amounts of both wild type and Dts36 virion extract. The

level of RNA guanylation was comparable between wild type and Dts36 under both 31°C and 40°C conditions. To more accurately compare the transfer of $\alpha^{32}\text{P}$ -GTP onto the RNA substrate, the individual bands were quantified by phosphorimager. The phosphorimager counts were graphed and are shown in Figure 4-1B. There is no detectable difference in guanylyltransferase activity between Dts36 and wild type at either 31°C or 40°C.

This experiment was repeated at 40°C with the same extracts. The level of guanylyltransferase activity is again comparable between wild type and Dts36. In this instance, Dts36 displays a slight decrease in activity but the difference appears to be minimal (data not shown).

In order to accurately compare guanylyltransferase activity, the amount of D1 protein in wild type and Dts36 extracts has to be similar. To determine the level of D1 protein in the viral extracts, increasing amounts of both wild type and Dts36 extracts were separated on an SDS-PAGE gel and immunoblotted with an anti-D1 antibody. The western blot (Figure 4-2) indicates that there is slight increase in the level of D1 protein in Dts36. This could indicate that there is less guanylyltransferase per D1 protein in Dts36 when compared to wild type. It is not unreasonable that Dts36 guanylyltransferase activity is actually lower than wild type for reasons which will be discussed later.

In conclusion, the guanylyltransferase is active in Dts36 and this evidence suggests that it is operating on a similar level to that found in wild type. Furthermore, the Dts36 guanylyltransferase appears to remain active at a high level regardless of the incubation temperature. However, the experiment will have to be repeated in a fashion

where only a single RNA product is observed. This is necessary for an accurate comparison between wild type and Dts36 activity.

These findings re-enforce the hypothesis that an impaired subunit interaction is responsible for the phenotype observed in Dts36.

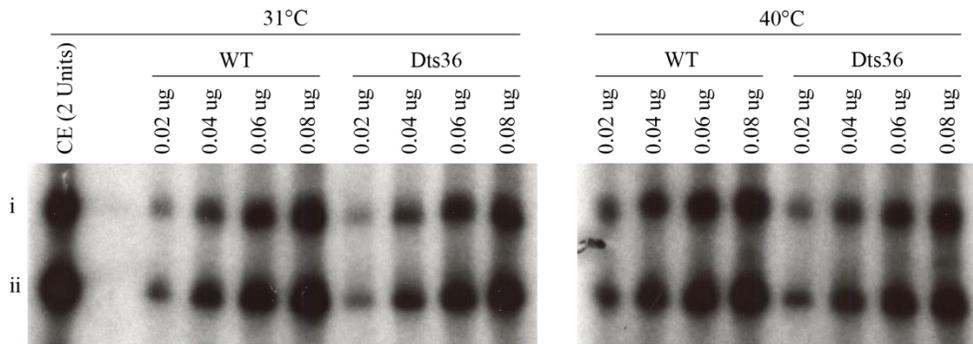
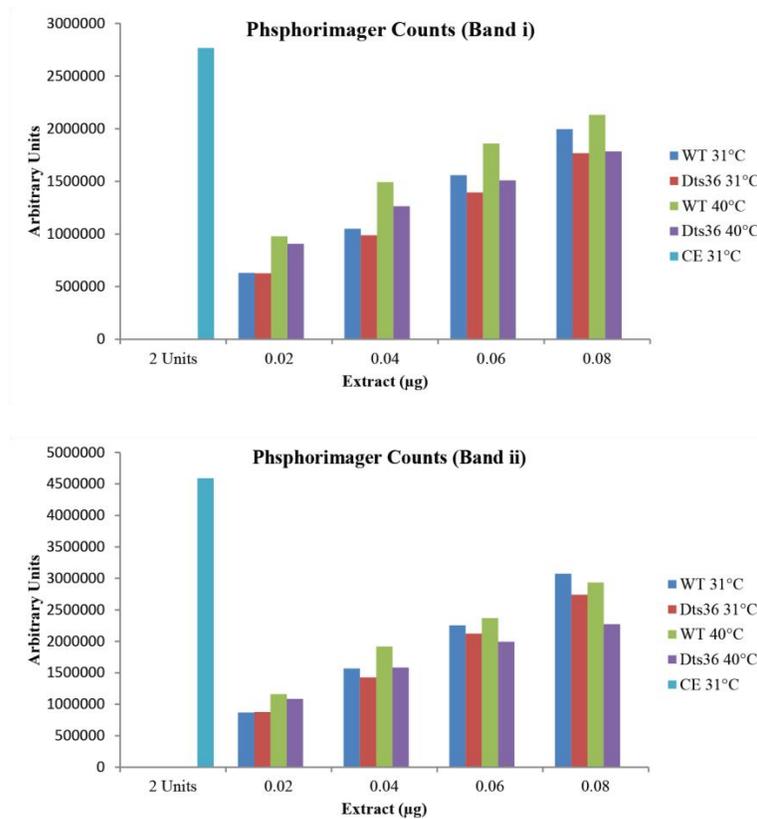
A**B**

Figure 4-1. *In vitro* guanylyltransferase assay of wild type (WT) and Dts36 purified virion extracts performed at 31°C or 40°C. (A) Autoradiographs of the urea-polyacrylamide gels. Level of purified virion extract (in µg) is designated directly above the autoradiographs. CE (pure capping enzyme) is commercially available purified vaccinia virus capping enzyme (strain WR). Band designation is located on the left. (B) Phosphorimager quantification of the individual bands (i & ii) found in A. Virion extract and incubation conditions are indicated on the right.

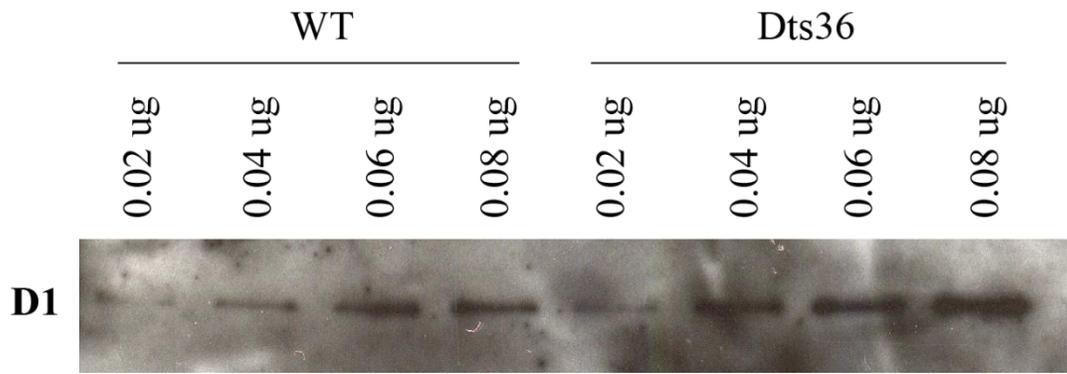


Figure 4-2. Western blot analysis of wild type (WT) and Dts36 viral extracts. Increasing amounts of wild type or Dts36 viral extract were separated on a polyacrylamide gel and detected with an anti-D1 antibody. Viral extract and the amount loaded (μ g) is designated on top.

CHAPTER 5 DISCUSSION

Dts36 is a previously characterized temperature sensitive mutant in the large subunit, D1, of the multifunctional mRNA capping enzyme. Previous studies indicated that Dts36 is specifically impaired in early gene transcription termination and intermediate gene transcription initiation *in vivo* and (guanine-N7-) methyltransferase *in vitro* (Condit unpublished). Furthermore, Dts36 was shown to have decreased levels of some early mRNAs *in vivo* which is consistent with unstable RNA as a result of a deficient methyltransferase (30). Previous reports indicate that both D1 and D12 subunits are required for early gene transcription termination (24, 25) and intermediate gene transcription initiation *in vitro* (24). D1-D12 binding has also been shown to be a requirement for (guanine-N7-) methyltransferase activity both *in vitro* (79, 80) and *in vivo* (101). Dts36 is deficient in all three of these activities. We hypothesized that Dts36 is temperature sensitive due to a compromised subunit interaction. Assuming this hypothesis is correct, the increased temperature would cause either a conformational change or misfolding of the D1 protein leading to a defective subunit interaction. More importantly, this has implications for the mechanisms of intermediate gene transcription initiation and early gene transcription termination. In an effort to elucidate the underlying mechanism responsible for the thermo-sensitivity of mutant Dts36 infection, further biochemical characterization of the multifunctional mRNA capping enzyme was conducted.

In this study, evidence is presented indicating that the protein-protein binding interaction in the D1-D12 heterodimer is compromised in Dts36 *in vitro*. Consistent with our hypothesis, Dts36 capping enzyme subunits were found to have a 2-3 fold lower

binding capacity relative to wild type. The D1 and D12 proteins were synthesized *in vitro* in the absence of other viral factors which indicates that the interaction impairment specifically involves the D1-D12 heterodimer. However, it is important to note that *in vitro* results do not necessarily translate *in vivo*. An *in vivo* immunoprecipitation study is complicated by the fact that the capping enzyme is associated with large ternary complexes and binds both to RNA (78) and the RNA polymerase (94). It is likely that the capping enzyme subunits would be stabilized by the ternary complex and obscure the binding deficiency. *In vivo* experiments were attempted, however efforts to liberate the capping enzyme from infected/transfected cell lysate failed as both D1 and D12 consistently sedimented with the pellet. Minimal liberation was only seen when using very harsh cell lysis buffers (data not shown). It is possible that D1 and D12 are sequestered in viral factories which were removed by centrifugation during preparation. However, treatment of lysates with nucleases which previously have been shown to break up the viral factories (24) failed to liberate the capping enzyme subunits (data not shown). This could indicate the D1 and D12 are mostly bound to larger order complexes.

Analysis of Dts36 D1 subunit model created in SWISS-MODEL suggests strong repulsive forces between the terminal oxygens of the mutated aspartate residue with backbone oxygens found within the same β -sheet as well as the adjacent β -sheet. This observation along with the fact that the mutated residue is well removed from the D1-D12 interface suggests that the binding deficiency seen in the co-immunoprecipitation experiments is the result of a conformational change in the D1 subunit. The D1-D12 interface involves a large area of contact with multiple hydrogen bonds and salt bridges

between the subunits (Figure 3-3). This type of interaction is characteristic of a strong protein-protein bond (91). Therefore, the mutated lesion in Dts36 D1 must be responsible for either a large or subtle conformational change to alter the D1-D12 interface.

The Dts36 G705D mutation location with regards to the methyltransferase domain is also revealing. Residue 705 is located in β -sheet 5 which is fairly conserved in methyltransferase enzymes from multiple species. An alignment of β -sheet 5 including *Homo sapiens*, *Xenopus laevis*, *Saccharomyces cerevisiae* and *E. cuniculi* shows 70-100% homology in 7 of the 12 amino acid residues. Glycine 705 specifically is 100% conserved across these species. More globally, β -sheet 5 is part of a highly ordered seven stranded β -sheet which is characteristic of the class I methyltransferase family (91). This indicates that the Asp705 residue is generally important for enzyme function. However this does not indicate whether the residue is important for the methyltransferase structurally or for catalytic function. The methyltransferase activity is the most extensively studied activity of the vaccinia capping enzyme and the large majority of catalytic residues have been identified (87, 89, 90). The crystal structure of the wild type (WR) C-terminal domain of D1 subunit bound to S-adenosylhomocysteine (SAH) is shown in Figure 5-1. The essential residues for catalysis in the image are shown in green and the GLY705 residue is shown in pink. The GLY705 residue is far removed from the enzyme active pocket indicating that GLY705 is important for the enzyme structurally. The conservation of residue 705 along with its position relative to the methyltransferase active site re-enforce the notion that the lesion in Dts36 is causing a conformational change in D1. Furthermore, these observations are consistent

with the idea that the D1-D12 subunit impairment seen in Figure 3-1 and Figure 3-2 is the result of the Dts36 lesion causing a conformational change in D1.

This region of the D1 protein has been analyzed previously for both methyltransferase activity and subunit interaction. *In vitro* analysis of a triple mutant, G704A, G705A, V707A, in β -sheet #5 of the D1 protein revealed a compromised D1-D12 interaction along with deficient methyltransferase activity. (80). One of the mutations is in the same amino acid as Dts36 (G705A vs. G705D). These results validate our own findings that Dts36 has an impaired capping enzyme subunit interaction. Furthermore, this evidence corroborates previous findings that Dts36 methyltransferase is defective *in vitro* (R. Condit unpublished).

Dts36 has previously been shown to have slight early termination slippage at lower temperatures which is increased at higher temperatures (30). Furthermore, methyltransferase is deficient at both permissive and non-permissive temperatures *in vitro* (R. Condit Unpublished). This evidence indicates a scenario where subunit interaction is somewhat compromised at lower temperatures and greatly exacerbated at higher temperatures. Experiments were performed to determine if higher temperatures would further alter Dts36 capping enzyme subunit interaction. Surprisingly, increased temperature had no effect on D1-D12 binding *in vitro* (Figure 3-2). It is possible that increased temperature has an effect on D1 folding during synthesis rather than instigating a larger conformational change. Experiments to test this were attempted *in vitro* however the rabbit reticulocyte lysate is not suited to this task. A different system will have to be explored in order to answer this question.

Assuming a D1-D12 interaction defect is the underlying cause responsible for the Dts36 phenotype, there are two mRNA capping activities which should remain unaffected. Both the RNA triphosphatase and guanylyltransferase enzymes are active in the absence of D12 (77, 78). Therefore, Dts36 should be capable of normal levels of both guanylyltransferase and RNA triphosphatase activities. Consistent with this hypothesis, evidence reported here suggests that the guanylyltransferase activity in Dts36 D1 is functional *in vitro*. Furthermore, Dts36 guanylyltransferase remains active at high levels *in vitro* even when incubated at temperatures which incapacitate the virus *in vivo*. The level of Dts36 guanylyltransferase appears to be comparable to wild type (Figure 4-1), however the amount of D1 protein found in the extracts is not identical (Figure 4-2). It appears that the Dts36 extract has a slightly higher concentration of D1 protein which would indicate that in fact Dts36 guanylyltransferase is somewhat impaired relative to wild type. This conclusion is reasonable and in fact expected with the knowledge that the Dts36 methyltransferase is inactive *in vitro*. As outlined in the introduction, the methyltransferase reaction is the only irreversible reaction catalyzed by the capping enzyme. As a result of a deficient methyltransferase, there is no mechanism in place to prevent reversal of the guanylyltransferase or RNA triphosphatase reactions. Furthermore, the guanylylation of mRNA caps has been shown to be both inhibited and reversed in the presence of pyrophosphate *in vitro* (68). Pyrophosphate release is the result of the guanylyltransferase enzyme removing the two terminal phosphates from the initiating RNA nucleoside (Figure 1-3, reaction two). Therefore, the level of guanylylated transcripts produced in the presence of Dts36

extract may be lower than that of wild type. However, this is mostly likely a result of capping reaction reversal as opposed to guanylyltransferase activity impairment.

An anomaly of the guanylyltransferase assay is the fact that multiple RNA bands appear in the reaction during the incubation. Unfortunately, the presence of multiple RNA bands makes an accurate comparison between wild type and Dts36 impossible. The presence of other smaller RNA products that have run off of the gel is likely. There are two possible sources for error that could result in multiple RNA bands. The likely possibility is that the RNA substrate was not gel purified prior to the guanylyltransferase assay. It is possible that multiple bands were synthesized during the *in vitro* transcription reaction and were then subjected to the assay. The other possibility is RNA degradation as a result of high concentrations of magnesium and high temperature. This phenomenon has been previously demonstrated. More specifically, RNA degradation has been observed in concentrations as low as 1mM magnesium. RNA degradation has also been shown in Tris-HCl buffers when the pH is over 7.5 and temperature is above 37°C (107). The reaction conditions used in the guanylyltransferase assay were 2mM magnesium in Tris-HCl pH 8.0 at temperatures of 31°C and 40°C. The guanylyltransferase assay conditions fall within this range. Therefore, it is a distinct possibility that the multiple RNA bands are the result of RNA degradation mediated by high magnesium concentration and heat.

Regardless of the technical issues, the Dts36 guanylyltransferase enzyme is active which is consistent with our hypothesis. This experiment will have to be repeated in order to accurately determine the enzyme's activity relative to wild type.

The results presented here corroborate our hypothesis that Dts36 is temperature sensitive as a result of a deficient D1-D12 subunit interaction in the vaccinia mRNA capping enzyme. More specifically, the *in vitro* co-immunoprecipitation results indicate that D1-D12 binding is specifically impaired and the guanylyltransferase assay results indicate that Dts36 guanylyltransferase is unaffected. Subunit interaction impairment is an appropriate mechanism for thermo-sensitivity in Dts36 considering the subunit requirements for three unrelated capping enzyme activities and the fact that Dts36 is specifically impaired in these activities alone. More importantly, these results are the first indication that subunit interaction is required for both early gene transcription termination and intermediate transcription initiation *in vivo* and therefore have mechanistic implications for these activities.

To validate the findings here, future experiments have to be performed. It is important to determine the capping status of vaccinia mRNA to determine if the effects seen *in vitro* are the same as what is occurring *in vivo*. Assuming that viral mRNAs are guanylated normally, this experiment would forgo the need to test RNA triphosphatase activity as the triphosphatase reaction must happen prior to the guanylyltransferase step. Furthermore, it is important to reproduce the early termination and intermediate initiation defects *in vitro* in order to prove that the mutant capping enzyme is specifically responsible.

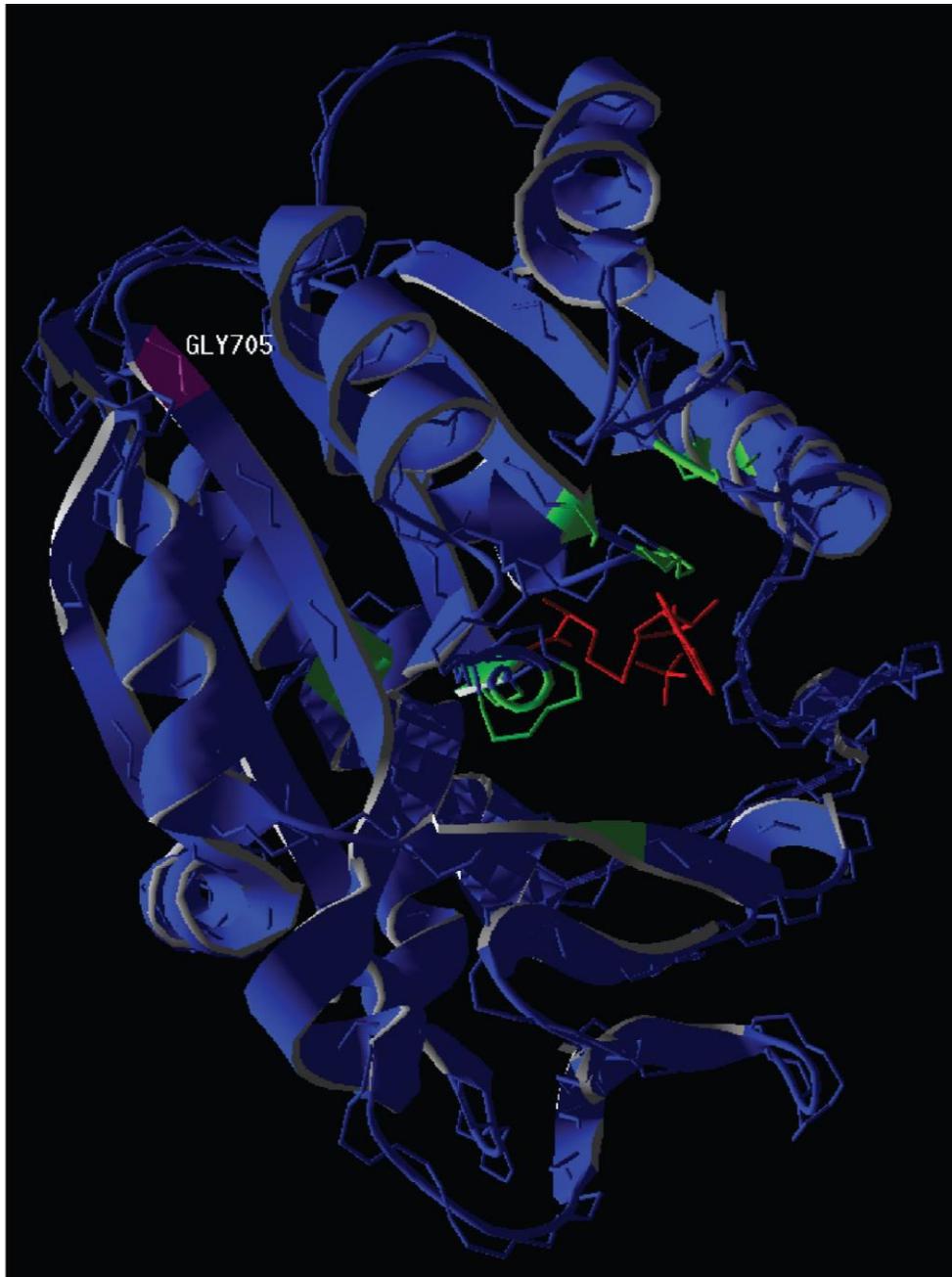


Figure 5-1. Crystal structure of the C-terminal domain of wild type (WR strain) D1 interacting with S-adenosylhomocystein (SAH). D1 is shown in blue with the residue 705 (mutant residue in Dts36) shown in pink. SAH is depicted in red and the D1 residues which are known to interact with SAH are shown in green. (Adapted from (91)).

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BIOGRAPHICAL SKETCH

Baron McFadden was born in Edmonton, Alberta, Canada in 1985. He completed a bachelor's of science in 2008 with an emphasis on molecular biology and biotechnology and a minor in biochemistry at University of Waterloo. He joined Dr. Condit's lab in 2009 and worked as an OPS technician conducting research for two years. He graduated with a master of science in translational biotechnology in 2013. In the future he plans to work in biotechnology.