CHARACTERIZATION OF TEMPERATURE SENSITIVE VACCINIA VIRUS MUTANTS FROM THE A3L AND E6R COMPLEMENTATION GROUPS

By

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Vaccinia virus temperature sensitive (ts) mutants provide actionable models for genetic study. By determining the growth and replicative phenotypes of the ts mutants, genetic function can be elucidated. After the mutagenesis of wild type virus, the newly created ts mutant viruses were assayed for protein synthesis and DNA replication. Those viruses that were normal for both were designated as having a normal phenotype. Normal phenotype mutants, while positive for protein synthesis and DNA replication, still have a condition that renders them defective for viral replication at the non-permissive temperature. The last stage of the viral replication cycle that can be investigated for defect is the morphogenesis cycle. A3L mutants and E6R mutants were investigated as candidates for morphogenesis research.

The A3L gene encodes for the precursor of the 4b major core protein. This protein comprises 11% of virion mass and is localized to the outer core wall of the virus. Characterization of the A3L mutant viruses, Cts8 and Cts26, was continued from work

done by Dr. Sayuri Kato which had shown that 1) the viruses had a normal protein and DNA phenotype, 2) the mutant viruses mapped to the A3L gene of the vaccinia genome, and 3) electron microscopy of the viruses showed a defective step in viral assembly. Analysis of the protein composition of the purified viral particles demonstrated that the mutant viral particles contain the same complement of proteins as wild type virus particles, including the protein synthesized by the defective A3L gene. Transcription analysis on purified particles showed that ts viral particles were unable to transcribe normally, but when transcriptional enzymes were extracted from viral cores, they performed as well as wild type enzymes. These results indicate that the A3L mutation disrupts the organization of enzymes within the core wall of the virions and that 4b is an essential protein for virion organization.

The E(2-8)a complementation group of ts vaccinia viruses was selected for study because the viruses were also classified as having the normal protein and DNA phenotype. Two different vaccinia strains are represented in this complementation group, Cts52, isolated by Dr. R. Condit, and Dts41 and 80, isolated by Dr. S. Dales. These morphogenesis candidates were mapped to the E6R gene of the vaccinia genome and the E6 genes from each were sequenced. The ts mutants were tested for protein synthesis and protein processing, and were also analyzed by electron microscopy. The results show that the viruses have normal protein synthesis and processing patterns, and that the viral particles appear to be assembled normally during an infection.

CHAPTER 1
INTRODUCTION

Vaccinia virus is the prototypical member of the Orthopoxvirus genus, Chorodopoxvirinae subfamily of poxviruses. It has no known natural host or reservoir but it has a wide host range in experimental systems (1). It holds a significant place in the study of virology since it was the first animal virus seen under a microscope; grown in culture, purified, titered and chemically analyzed (2); but most significantly, it was the virus used in the prophylactic vaccine that ended the reign of smallpox, variola, in 1977 (3). Its study continues to be relevant today for the wealth of information it holds about viral replication and structure. To elucidate the functions of the hundreds of proteins it encodes would take scientists many steps closer to better managing infections and disease. In studying temperature sensitive vaccinia virus mutants, we hope to discover the structure and function of genes that impact virus replication and development.

Vaccinia Virion and Genome Structure

Vaccinia is a large, complex virus particle that, under electron microscopy (EM), is rectangular or brick-shaped with a dense core. The virus exists in several distinct forms through its development (Figure 1.1). The infectious extracellular enveloped virus (EEV) form is composed of three lipid bilayers derived from host cell membranes during morphogenesis (4-6). Other infectious forms of the virus, cell-associated enveloped virus (CEV), intracellular enveloped virus (IEV), and intracellular mature virus (IMV) have three, four, and two lipid bilayers (6), respectively. Two structures, the lateral bodies, flank the viral core; they are trypsin-sensitive and are of unknown origin and function.

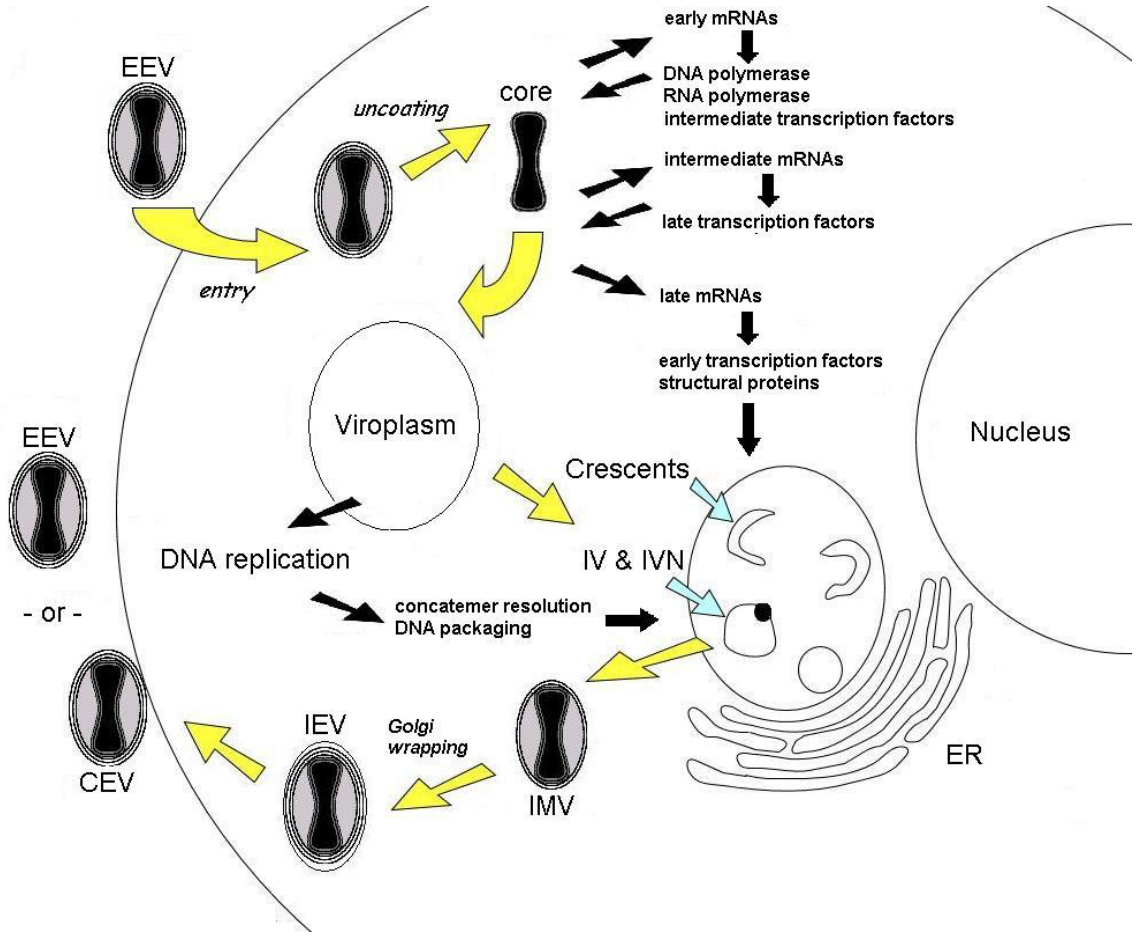

Figure 1.1. Vaccinia life cycle. Upon entering the cell, the virus will uncoat and begin synthesizing early mRNAs. Those gene products encode factors necessary for subsequent rounds of RNA transcription and DNA replication. DNA replication begins forming concatemeric segments of viral DNA. Intermediate transcription begins after DNA replication and those transcription products encode for late transcription factors. Late transcription products encode for structural proteins and early transcription factors that are packaged into the assembled virions. Assembly begins with the appearance of crescents derived from the cellular ER. The crescents enclose viroplasm and become IVs until concatemeric DNA is resolved and packaged into them, making them IVNs. IVNs exit the viroplasm and mature to form IMVs. IMVs are wrapped by the golgi complex to become IEVs which are transported to the plasma membrane. The last of the lipid bilayers fuses with the plasma membrane leaving the virion outside the cell as a CEV or EEV.

The viral core is dumbbell-shaped and contains viral DNA and replicative enzymes packaged during morphogenesis. These enzymes are necessarily packaged into each viral core because the virus carries out its infection within the cytoplasm of the host cell and,

therefore, does not have access to host cell replicative machinery in the nucleus. The single, linear, double-stranded DNA of vaccinia is approximately 200 kb in length, depending on the vaccinia strain (7). The genome encodes roughly 200 intronless genes, 100 of which are associated with virion (8;9). Vaccinia DNA, like other poxviruses, has inverted terminal repeats (ITRs) at each end of the genome that vary in size from poxvirus to poxvirus (10-13). The ends of these ITRs contain hairpin loops that have a critical role in DNA replication.

Vaccinia Transcription and DNA Replication

Vaccinia, as a member of the poxviruses, undergoes its entire life cycle within the cytoplasm of the infected cell, it must, therefore, encode and/or carry all the proteins and enzymes required for RNA and DNA synthesis. Throughout its life cycle within the cytoplasm, vaccinia proceeds through a tightly regulated time course of synthesis and replication. There are three separate stages of RNA synthesis in a vaccinia infection, each with its own set of promoters. Early transcription precedes DNA replication, intermediate and late transcription. The virus must undergo a tightly regulated pattern of transcription, replication and viral assembly to have a successful infection (Figure 1.1).

The start of the viral replication cycle occurs when an infectious virion, an intracellular mature virus (IMV), cell-associated enveloped virus (CEV) or extracellular enveloped virus (EEV), recognizes and enters the host cell. The mechanism of viral entry, or viropexis, is, as yet, unknown, but there is evidence that there may be a cell-surface receptor involved since IMV particles have been shown to associate with membranes within vacuoles formed by cell-surface invaginations (14) and with the plasma membrane (5). Given vaccinia's wide host range, any theoretical receptor must be highly conserved but the exact nature of the receptor has yet to be discovered. Following uptake, the outer

membrane of the virus is shed leaving the virus cores within the cell cytoplasm. The fate of the uncoated membrane is unknown, but the susceptibility of viral DNA to deoxyribonuclease is biochemical evidence that an uncoating event happens (15). Transcription machinery packaged in the viral core begins early mRNA synthesis. Two forms of viral RNA polymerase exist during an infection, the first is a polymerase specific to genes that are transcribed early, while the second transcribes intermediate and late genes. The viral RNA polymerase is a eukaryotic-like, multi-subunit enzyme that is transcribed throughout infection (1). The early polymerase contains an extra subunit, RAP94. RAP94 is a 94-kd enzyme essential for early transcription that is encoded by the viral gene H4 late during infection (16;17) and packaged into progeny virions during morphogenesis as a subunit of the RNA polymerase. Early transcription products are homogeneous in sequence at their 5' and 3' ends and are capped and polyadenylated (18) like eukaryotic mRNAs. The core extrudes the early products and then uncoats within the cellular cytoplasm to form the focus of DNA replication, the virosome (19). This uncoating event is likely catalyzed by one of the early gene products because if protein synthesis inhibitors are added to an infection, the core is unable to uncoat (15). Early gene products are detectable 20 minutes after infection (1) and include factors involved with DNA replication, intermediate transcription and host cell interactions.

After early transcription and uncoating of the viral core, the E9 gene product, the viral DNA polymerase (20), and other factors, initiate DNA replication which results in concatemeric segments of DNA that must be resolved to form unit genomes that are subsequently packaged into the progeny virus cores. The process of DNA replication is also necessary in order for the intermediate stage of transcription to begin (21).

Only a few intermediate transcription products, detectable 100 minutes after infection, have been characterized. The few intermediate genes that have been analyzed encode, among other things, factors required for late transcription (22). Late transcription products, detectable 140 minutes post infection, continue to be synthesized through 48 hours post infection. Virion structural proteins and early transcription factors comprise the bulk of these late products. Their roles are primarily in the morphogenesis of viral particles and include proteins and enzymes that are packaged within developing virions for subsequent infections.

Vaccinia Morphogenesis

Following transcription and DNA replication the next stage of an infection is the assembly of new virus particles. Viral morphogenesis begins after transcription and DNA replication and is best studied by electron microscopy (EM) of cells fixed at various stages of infection. After a virus enters the cytoplasm of the cell and uncoats (15), the core of the virus is left to begin DNA replication in the cytoplasm of the cell (23). It is unclear whether the core has its own lipid bilayer that is also shed before DNA replication begins, but the core degenerates into the amorphous viroplasm, the site of DNA replication (24). The viroplasm excludes cellular structures (25) and is first seen 2-3 hours after infection. Viral assembly begins after late transcription with the appearance of distinct structures derived from the host cell's endoplasmic reticulum (6;26). These membrane cisternae, modified by viral proteins (27;28), collapse on themselves to create a double lipid bilayer that encircles a segment of viroplasm (6). The encapsulating cisterna is called a crescent, after its two dimensional appearance with EM (8;24), and encloses some of the viroplasm to form the spherical, immature virion (IV). Viral DNA is packaged into the immature virion as a nucleoid, and the particle undergoes further

differentiation to become the intracellular mature virion (IMV) (24;29). The viral DNA, along with the enzymes packaged with it, becomes the virus core while the flanking lateral bodies appear to give the core its dumbbell-shaped appearance. During the development of IV to IMV, the virion is transported away from the virosome by microtubules (30). The IMV precursor to a fully mature extracellular virus is fully infectious and is the primary form isolated during purification. Very few of the IMVs that are made proceed to the IEV stage of development when a modified Golgi membrane adds the last of the double lipid bilayers to IMV (31;32). The IEV, surrounded by four lipid bilayers, is transported to the cell membrane via microtubules where the last of the lipid bilayers fuses with the cell membrane leaving three lipid bilayers surrounding the extracellular virus. The virus may stay associated with the cell membrane to infect an adjacent cell as the cell-associated enveloped virus (CEV) (33), or it may dissociate from the host cell membrane to become the extracellular enveloped virus (EEV), free to infect another cell and begin the cycle again (34).

Temperature Sensitive Mutant Collections

In order to analyze the functional organization of the vaccinia genome, several laboratories have isolated temperature sensitive (ts) mutant viruses through various methods of mutagenesis. Temperature sensitive viruses are able to grow at the lower, permissive temperature of 31ºC, but not at a higher, or non-permissive temperature of 40ºC (35). Although other types of mutant viruses are made during mutagenesis, ts viruses are highly desirable for experimental analysis because of their conditional lethal phenotype and because any essential gene can, theoretically, be mutated to create a temperature sensitive mutant. The ts phenotype can be mapped to a gene, helping to catalog the functions of the hundreds of genes present in the genome. To construct the

library of temperature sensitive mutant vaccinia viruses, Drs. Richard Condit and Samuel Dales, in separate laboratories, mutagenized wild type virus. After screening the viruses for temperature sensitivity, the viruses were assayed for DNA and protein synthesis and for virus morphogenesis under EM (8;36;37) yielding the Condit temperature sensitive viruses (Cts) and Dales temperature sensitive viruses (Dts).

Each virus's ability to synthesize DNA was assayed by pulse labeling with [^{3}H] thymidine. Viruses were also assayed for a time course of viral protein synthesis using a protein pulse labeling assay with [^{35}S] methionine, processing the samples by SDS-PAGE and visualizing the radioactive signals with autoradiograms. Viruses isolated by the Dales laboratory were analyzed by EM and categorized by their appearance.

Four different temperature sensitive phenotypes were observed in the DNA and protein synthesis experiments: DNA negative, defective late, abortive late, and normal. Mutant viruses that were unable to synthesize DNA during infection at the non-permissive temperature, 40ºC, were called "DNA-negative" viruses. DNA negative viruses, while able to synthesize early viral proteins and shut off host protein synthesis normally, cannot make DNA, and therefore, will not progress to the intermediate and late protein synthesis stages of replication. The "defective late" phenotype is characterized by the slowed and/or delayed synthesis of late viral proteins at 40ºC, and may also include slowed shut off of host protein synthesis and slowed early viral protein synthesis. Viruses with the "abortive late" phenotype will proceed through the steps of infection, including host protein shut off and early viral protein synthesis, but once late translation has been initiated, the synthesis of these late proteins stops (36-38). Without the late transcription products, the virus cannot continue through morphogenesis and so the infection ends in

the infected cell and cannot spread. The “normal” phenotype is characterized by normal DNA and protein synthesis at the non-permissive temperature like wild type virus, suggesting that the mutation making the virus temperature sensitive affects a different developmental stage of the virus. This condition makes mutant viruses with normal phenotypes candidates for the study of morphogenesis.

Using cosmid clones in marker rescue experiments, Condit and co-workers were able to preliminarily map several of the Cts mutant viruses that were isolated. In subsequent collaboration with the ATCC, mutant viruses isolated by Dales and co-workers were added to the collection held by the Condit laboratory and all of the viruses were subjected to an extensive complementation analysis (39). This complementation analysis was used to determine if some viruses shared mutations in the same genes. Viruses that can aid each other’s growth during co-infection under non-permissive conditions are said to be “complementing.” When viruses are able to complement each other and grow under non-permissive conditions, the mutations are in different genes and the viruses are, therefore, not in the same “complementation group.” If the viruses each have a defective copy of the same gene, neither will produce a viable gene product and neither virus can grow under the non-permissive condition; the viruses are said to be “non-complementing” and in the same “complementation group.” The study by Lackner et al. yielded 53 different complementation groups containing 132 temperature sensitive mutants of vaccinia from both the Condit and Dales collections (39).

The isolated mutant viruses, separated by phenotype and complementation group, are important models for studying viral gene function. The temperature sensitive mutants

in the A3L and E6R complementation groups were selected specifically as candidates for morphogenesis research because of their normal protein and DNA phenotypes.

The A3L gene of vaccinia encodes a 72.5 kDa protein that is made late during infection. The protein, p4b, is proteolytically processed to yield a 62 kDa protein, 4b, that is found in the outer core wall and comprises 11% of total virus mass. The A3L mutants, Cts 8 and Cts26, were originally mapped by marker rescue to the vaccinia genome between the right end of the HindIII D restriction enzyme fragment and left side of the HindIII A restriction enzyme fragment (37;40). More recently, the mutant viruses were mapped to the A3L gene by marker rescue and sequenced by Kato et al., in preparation. A T-to-C transition occurred in Cts8 changing an alanine to a valine at residue 562, and two C-to-T transitions occurred in Cts26 changing codons 192 and 341 from serines to phenylalanines. The normal protein synthesis and processing phenotypes of both viruses were confirmed by protein pulse and pulse-chase experiments that showed that the protein synthesis patterns of both mutant viruses were indistinguishable from wild type. Similarly, when DNA replication and concatemeric DNA resolution were assayed, the mutant viruses showed no differences when compared to wild type. Thus, both mutants appeared normal for every developmental process except morphogenesis. EM analysis of infections with the A3L mutant viruses demonstrated aberrant forms of the IMV at the non-permissive temperature. Wild type IMV, as demonstrated in Figure 1.1, has the brick or dumbbell shaped core that is dense and flanked by lateral bodies. The virions are spherical in shape and appear symmetrical no matter how they are sectioned for EM. In the aberrant particles, the core is not dumbbell or brick shaped, but rather contorted

within the irregular envelope of the virion. Aberrant cores are not anchored in the center of the virion (Figure 1.2) like the wild type cores and dense material is scattered

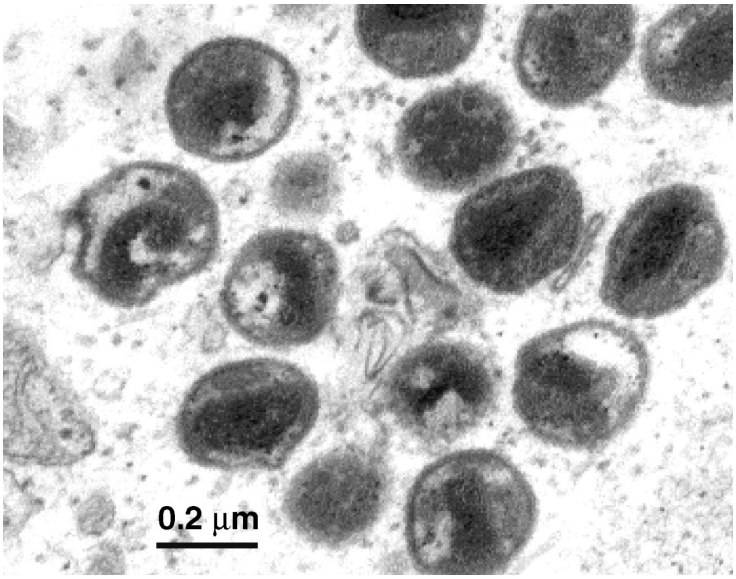

Figure 1.2. Cts8 aberrant IMV particles. Cells were infected with Cts8, grown at 40°C and fixed for EM. The virions above are aberrant IMVs (Kato et al, in preparation).

around the particle, clouding the entire aberrant virion. Mutant viral particles are often not symmetrical, but lop-sided and disfigured. The differences observed by EM between a wild type particle and the mutant's aberrant particle are unmistakable. With this information about the phenotype, sequences and the aberrant particles of the A3L mutant viruses, experiments to determine the protein composition and transcription phenotypes of the mutant virions were started.

Review of the complementation analysis by Lackner et al., revealed another group of candidate viruses for morphogenesis research. The mutants of the E(2-8)a complementation group, Cts52, Dts41 and Dts80, were selected for characterization. Preliminary data on the Cts52 mutant performed by Condit and co-workers showed that it mapped to the E(2-8) region in the vaccinia genome. The normal protein and DNA

synthesis phenotypes of Cts52 were also discovered at that time. After the complementation analysis by Lackner et al., the Dts41 and Dts80 mutants were grouped along with Cts52 in the E(2-8)a complementation group. EM data of Dts41 and Dts80 from Dales and co-workers, showed that the viruses produced normal or nearly normal particles at the non-permissive temperature (8). Using this information, the process of characterizing this group of three ts vaccinia viruses was started.

CHAPTER 2
MATERIALS AND METHODS

Cells and Virus

African green monkey kidney cells (BSC40), viruses Cts52, Dts41 and Dts80, and the conditions for cell culture, vaccinia virus growth, infections and plaque titration are as described in Condit et al., 1983, Condit & Motyczka, 1981, and Dales & McFadden, 1977 (8;36;37). Condit and co-workers used the Western Reserve (WR) vaccinia strain in the isolation and analysis of Cts mutant viruses while Dales and co-workers used the IHD-W vaccinia strain in mutant isolation and analysis.

Protein Pulse Labeling

To determine the protein synthesis phenotype of the mutant viruses, BSC40 cells were grown to confluency in 60 mm dishes. Cells were infected with each of the four viruses: wild type WR strain, Cts52, Dts41 and Dts80, at a multiplicity of infection (MOI) of 10 in PBS. Infections were carried out at 31ºC and 40ºC for 30 minutes, rocking dishes every 15 minutes to distribute the inoculum over the monolayers. Inoculum was aspirated from the plates and prewarmed media was added to the dishes. At 0, 3, 6, 9, 12 and 24 hours after infection, media was aspirated from cells. Cells were washed once with prewarmed PBS and then overlaid with 0.5 ml of PBS containing [^{35}S] methionine (20 μCi/ml). The cells were incubated at the appropriate temperature for 15 minutes and the labeled methionine was removed. Immediately, 300 μl of 1X Laemmli buffer (50 mM Tris-Cl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) was applied to the cells. With a rubber policeman, cells were scraped from the

dish and transferred to labeled ependorf tubes. Samples were boiled for 4 minutes, frozen and reboiled to reduce viscosity. 25 µl of each sample was loaded onto a 10% acrylamide-SDS minigel. Gels were run at 120V until dye front reached the end of the minigel. The gels were stained in Coomassie stain and then destained, soaked in 7% acetic acid overnight, dried and autoradiographed (37).

Protein Pulse-Chase Labeling

To determine the protein processing phenotype of the mutant viruses, BSC40 cells were grown to confluency in 60 mm dishes. Cells were infected with virus in PBS at an MOI of 10 for 45 minutes at either 31°C or 40°C. After 45 minutes, inoculum was aspirated and 2 ml prewarmed media was added to the infected monolayer. The cells were then incubated at the appropriate temperature for eight hours. After eight hours of infection, defined as T=0, media was aspirated from the dishes, cells were washed two times with 0.5 ml prewarmed PBS and then overlaid with 0.5 ml PBS containing [^{35}S] methionine (20 µCi/ml) and incubated for 15 minutes. Label was removed from monolayers; cells were washed once with 1 ml of prewarmed media, then overlaid with 2 ml prewarmed media and incubated at appropriate temperature for various times. At 0, 2, 8, or 16 hours after labeling, media was removed from cells and 300 µl of Laemmli buffer was added to each dish. Rubber policemen were used to scrape cells into labeled ependorf tubes. Samples were boiled, frozen and boiled to reduce viscosity. 25 µl of each sample was loaded onto a 12% acrylamide-SDS gel. Gels were run at 120V until dye front reached end of the minigel. Gels were stained in Coomassie, destained, dried and autoradiographed.

Viral DNA Isolation From Infected Cells

To purify viral DNA, 150 mm dishes of BSC40 cells were grown to confluency and infected with 4 ml virus diluted in PBS inoculum at a MOI of 0.1. After a 45 minute incubation at 31^{o}C, the inoculum was aspirated and prewarmed media was added back to the cells. The dishes were incubated at 31^{o}C until full cytopathic effect (CPE) was observed. Cells and media were harvested into 50 ml conical tube and centrifuged at 1000 rpm in a J6B rotor for 10 minutes. The supernatant was discarded and the pellet was resuspended in 20 ml of isotonic buffer (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 5 mM EDTA). Cells were centrifuged at 2000 rpm in a Sorvall RT6000 centrifuge for 5 minutes at 4 ^{o}C. After the supernatant was aspirated and discarded, the pellets were resuspended in 9 ml of hypotonic buffer (10 mM Tric-HCl, pH 8.0; 10 mM KCl; 5 mM EDTA), and incubated on ice for 10 minutes. 25 µl of beta-mercaptoethanol and 1 ml of 10% Triton X-100 were added and sample was spun in Sorvall RT6000 at 2000 rpm for 5 minutes. The supernatant was decanted into a 15 ml conical tube and spun in a Sorvall centrifuge as before. Supernatant was decanted into a 25 ml screw-top plastic corex tube and spun at 9000 rpm for 30 minutes in a Sorvall SA600 rotor to pellet viral cores. The supernatant was decanted and discarded while the pellet was resuspended in 4.5 ml TE in a 50 ml conical tube. 15 µl of beta-mercaptoethanol, 50 µl of Proteinase K, 200 µl of 5 M NaCl, and 500 µl 10% SDS was added to sample and incubated at 37 ^{o}C for 30-120 minutes. DNA was extracted two times with equal volumes of phenol-STE. The DNA was precipitated with 2.5 volumes of 100% ethanol. With a heat sealed Pasteur pipette, the precipitated DNA was collected and washed in 70% ethanol. The DNA was allowed to dry on the pipette and then resuspended overnight in 100 µl TE (41).

Terminal Dilution

To determine the amount of virus to be used in one step marker rescue experiments, confluent 60 mm dishes of BSC40 cells were infected with 1:3 dilutions of virus in PBSAM (PBS: 170mM NaCl, 3.35mM KCl, 10mM Na_2HPO_4, 1.84mM KH_2PO_4; PBSAM includes 0.01% BSA and 10mM $MgCl_2$), starting with a 1:100 dilution. Cells and inoculum were incubated at the non-permissive temperature for a total of 1 hour while rocking the plates every 15 minutes. The inoculum was removed and pre-warmed media was added to the cells before replacing them at the non-permissive temperature. After four days at 40ºC, the cells were stained with crystal violet. The dilution used in one-step marker rescue experiments was one that fell between dilutions that disrupted the monolayer of cells and left the cells intact (40).

Polymerase Chain Reactions

To map the mutant viruses by marker rescue and sequence the mutant genes, the primers that were used for polymerase chain reactions (PCR) were constructed using the Vector NTI database and ordered from Sigma Genosys. The primer specifications were used to optimize the thermocycler protocols. For use in marker rescue, five PCR primer pairs developed by Dr. Ben Luttge were used to create five 5 kb products that span the E(2-8) genes and flanking regions of the vaccinia genome. For marker rescue transfection products, the thermocycler protocol that was used was 94 ^{o}C for 15 seconds, 53 ^{o}C for 30 seconds and 68 ^{o}C for 5 minutes. For sequencing, the program designed for the thermocycler was 94ºC for 15 seconds, 50ºC for 30 seconds, and 72ºC for 3 minutes, all for 10 cycles and then 94ºC for 15 seconds, 50ºC for 30 seconds, and 72ºC for 3 minutes and 30 seconds, for 30 cycles. After detecting products of the correct length by electrophoresis on a 0.6% LE agarose gel, samples were loaded onto Microcon brand

filters in ependorf tubes and centrifuged at 500 G for 15 minutes. The effluent was discarded and water was added to the filter to resuspend the product; the filter was inverted over a new ependorf tube and centrifuged at 1000 G for 2 minutes. The concentration of the DNA was determined by OD_{260}.

One Step Marker Rescue with Genomic DNA

To determine the map location of the temperature sensitive mutants on the viral genome, 60 mm dishes were grown to confluency. Media was removed from the cells and 0.5 ml of PBSAM containing a concentration of virus determined by terminal dilution was added. Inoculum was added to the monolayers and dishes were incubated at the non-permissive temperature, 40 ^{o}C, for one hour. Inoculum was removed from the cells and pre-warmed, serum-free media was added to the cells. Cells were transfected with a 100 µl solution containing a 50 µl solution of 1 µg of PCR amplified DNA, 100 ng of genomic DNA and water which was mixed with a 50 µl solution of 30 µg of lipofectin and water. Dishes were replaced at the non-permissive temperature overnight, media on the cells was changed to the standard 1X DME/10% FCS and cells were incubated for an additional three days. Dishes were then removed from the incubator and cells were stained with crystal violet (37).

Sequencing

To determine the exact nature of the mutations in the mutant viruses, a region of DNA larger than, and containing the E6 region of each virus was PCR amplified. The ORF-specific primers, E5 forward and E7 reverse, were used generating a 3.341 kb product. For sequencing primers, seven different forward-reading primers were designed to begin outside the E6 gene and read across the gene in overlapping products. The first of these products was designed to begin before the start of E6 in order to avoid

constructing a contig with primer derived sequence rather than mutant derived sequence. Sequencing was performed at the University of Florida Sequencing Core Laboratory at Fifield Hall.

Virus Particle Purification

To isolate viral particles for analysis, confluent 150 mm dishes of BSC40 cells were infected with virus, Cts8, Cts26 and wt (wt40 ^{o}C), MOI of 10, and incubated at the non-permissive temperature. Another preparation of wild type (wt37 ^{o}C), MOI 0.1, was grown at 37 ^{o}C. Infected cells were collected after 24 hours while wt37 ^{o}C infected cells were collected after a two day incubation. Samples were centrifuged in 50 ml conical tubes in a Sorvall RT6000 centrifuge at 2000 rpm for 5 minutes at 4 ^{o}C. The supernatant was discarded and pellets were resuspended in 10 ml 10 mM Tris pH 8.0. Samples were dounce homogenized gently for 20 strokes and centrifuged again in the Sorvall RT6000 centrifuge. The supernatant was removed into a new 50 ml conical and the pellet was resuspended in 5 ml 10 mM Tris pH 8.0, dounce homogenized and spun as before. This procedure was repeated once more, and all the supernatants collected were pooled. The 20 ml of pooled supernatant was centrifuged in the Sorvall RT6000 centrifuge at 2000 rpm for 10 minutes at 4 ^{o}C to remove remaining nuclei. The pooled supernatants were removed to a new 50 ml conical tube and sonicated for 15 seconds eight times. Sonicated product was layered onto two 16 ml 36% sucrose cushions and centrifuged in an SW28 rotor at 18,000 rpm for 80 minutes at 4 ^{o}C to remove cellular debris. The supernatant was decanted and discarded while the pellet was resuspended in 3 ml 10 mM Tris pH 8.0 and sonicated again. The product was layered onto a 33 ml 25-40% sucrose gradient in 10 mM Tris-HCl pH 8.0 and centrifuged in the SW28 rotor at 13,500 rpm for 40 minutes at 4 ^{o}C. The purified virus formed a band within the gradient and was carefully removed and

diluted with two volumes of 10 mM Tris pH 8.0 in an ultra-clear Beckman centrifuge tube. The tubes were centrifuged at 15,000 rpm for 60 minutes at 4 ^{o}C to pellet the viral particles. The supernatant was decanted and discarded and the purified virus pellet was resuspended in 2 ml 10 mM Tris pH 8.0. The OD_{260} was read on a 10 µl sample suspended in 300 µl 10mM Tris pH 8.0 and the result was converted into particles of virus per ml for use in subsequent experiments. Standards used in the calculation include the following: 1 OD_{260} = 1.2 X 10^{10} virus particles/ml; 1 OD/ml concentration of virus = 0.067 mg/ml; 1 mg virus = 1.77 x 10^{11} virus particles (42).

Electron Microscopy

To observe the stages of viral morphogenesis in the mutant virus infections, 60 mm dishes of confluent BSC40 cells were infected with virus (MOI of 10) diluted in PBSAM. Infections were incubated at the non-permissive temperature for 45 minutes and the inoculum was removed. Prewarmed media was added to the cells and they were allowed to incubate at the non-permissive temperature of 40ºC for either 24 or 48 more hours. After incubation, the media was removed and cells were washed with a 0.1 M sodium cacodylate + 1 mM $CaCl_2$ buffer, pH 7.24. After the buffer was removed, a 2% gluteraldehyde solution in sodium cacodylate buffer was added to the cells and incubated for 1 hour at room temperature. The dishes were rocked occasionally to distribute the buffer evenly over the cells to prevent them from drying. The cells were collected and centrifuged at 2800 rpm for 2 minutes. The supernatant was removed and the pellet was resuspended in sodium cacodylate buffer. The samples were taken to the electron microscopy core facility at the University of Florida for processing.

Transcription by Permeabilized Virions

To assay virion transcription by mutant virions, 250 µl reactions were prepared, each containing 0.25 OD_{260} units of purified virus, 0.25 M Tris, 50 mM DTT, 50 mM $MgCl_2$, 25 mM ATP, 5 mM UTP, 5 mM GTP, 0.25 mM CTP, and 5 µCi α^{32}P-CTP. After being mixed on ice, a 50µl sample was removed into 5% TCA on ice. The remainder was incubated in a 37 ^{o}C water bath with 50 µl samples removed to cold 5% TCA at 30, 60 and 90 minutes. Precipitated material was collected on glass fiber filters and counted in a liquid scintillation counter.

Western Blot Analysis

To determine the protein composition of the purified mutant virus virions, purified virus samples were processed by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked twice with a solution of PBS, 0.05% Tween-20 (PBS-T), and 5% non-fat dry milk (PBS-T/NFDM) for 30 minutes and then incubated with an appropriate concentration of primary antibody in PBS-T/NFDM for one hour. The membranes were rinsed and washed three times in PBS-T and incubated with the appropriate secondary antibody in PBS-T/NFDM for one hour. The membranes were rinsed and washed again in PBS-T and then incubated with an enhanced chemiluminescence detection kit from Amersham as directed to visualize the signals.

Antibodies provided by Dr. Moss used in Western blot analysis include anti-H4 at a 1:5000 dilution and anti-A29 at a 1:500 dilution. Antibodies provided by Dr. Shuman include anti-J6 and -A29 used at 1:1000 dilutions. Dr. Hruby provided A10, L4 and A3 antibodies used at 1:1000 dilutions. Dr. Traktman provided an H5, H1 and F10 antibody used at 1:1000 dilutions. Dr. Niles provided a J3 antibody used at 1:5000; D11and D1 antibodies used at 1:1000; E1 and D8 antibodies used at 1:500. An F17 antibody, from

the Condit laboratory and an A18 antibody created by the Hybridoma core at the University of Florida were used at 1:1000 and 1:10,000 dilutions, respectively.

CHAPTER 3
RESULTS

A3L Mutant Viruses

Virus Purification

Virus was purified for analysis of protein composition by SDS-PAGE and Western blot as well as transcription ability of permeabilized virions. Two different purification preparations were used. In one preparation, cells were infected with a high MOI (10) of Cts8, Cts26 or wt virus and incubated at the non-permissive temperature for 24 hours. The purified wt virus from this infection was designated wt40ºC. Another wild type preparation of virus was made using a low MOI (0.1) infection, and incubating at 37ºC, yielding wt37ºC. Following infection, the virus from the cytoplasmic extracts of the infected cells was purified by differential centrifugation and sedimentation on sucrose gradients. The wild type particles, when sedimented on a sucrose gradient, banded in a 2 to 3 mm space two-thirds of the way down the gradient. After being pelleted and resuspended in 10 mM Tris pH 8.0, the yield of the wild type virus purification was calculated using an OD_{260} reading on a sample of the resuspended particles. The purified viruses were also assayed for infectivity by plaque titration at 31ºC. The wt 37ºC yield was 2,128 particles of virus per infected cell with a particle to infectivity ratio of 64 viral particles per plaque forming unit (pfu). The wt 40ºC yield was 622 particles per infected cell with a particle to infectivity ratio of 42 particles per pfu. The sedimentation profiles of the mutants were the same as the wt; both types of mutant virions banded in the same place on the gradient and with the same width as the wild type preparations. The Cts8

yield was 826 particles per cell and 4750 particle per pfu and the Cts26 yield was 419 particles per cell and 1260 particles per pfu. The wt37ºC particle per cell yield was five fold better than the Cts26 yield, but if the mutant virus yields are compared to the wt40ºC preparation, grown under the same conditions as the mutants, the yields do not differ significantly. Of note, mutant virus preparations were 100 times less infectious than the wild type preparations.

Total Protein Composition of Purified Particles

To compare the protein composition of the purified mutant virions to the wild type, samples of the purified virions were separated by SDS-PAGE and stained with Coomassie and silver stain (Figure 3.1). The protein composition of the wt40ºC virions shows a doublet at approximately 60 and 62 kDa which represents the 4a and 4b proteins, respectively. The largest doublet, best seen in the last three lanes, represents the polymerase subunits RPO 147 and RPO 132. Note that the 62 kDa band, representing the 4b protein, is present in both mutant virion profiles indicating that even though their mutation is in the gene that encodes for 4b, the protein is still made, it is processed and it is detectable in the virions. Both mutant virion preparations have the same protein composition profile as the wt40ºC virion preparation, with the exception of faint bands seen in the wild type virions at approximately 70 kDa. These bands were excised and analyzed by mass spectrometry, but the results were uninformative and further attempts to reproduce the pattern were unsuccessful. The conclusion reached from this assay was that, at this resolution, the mutant virions appear to contain the same proteins as wild type virions.

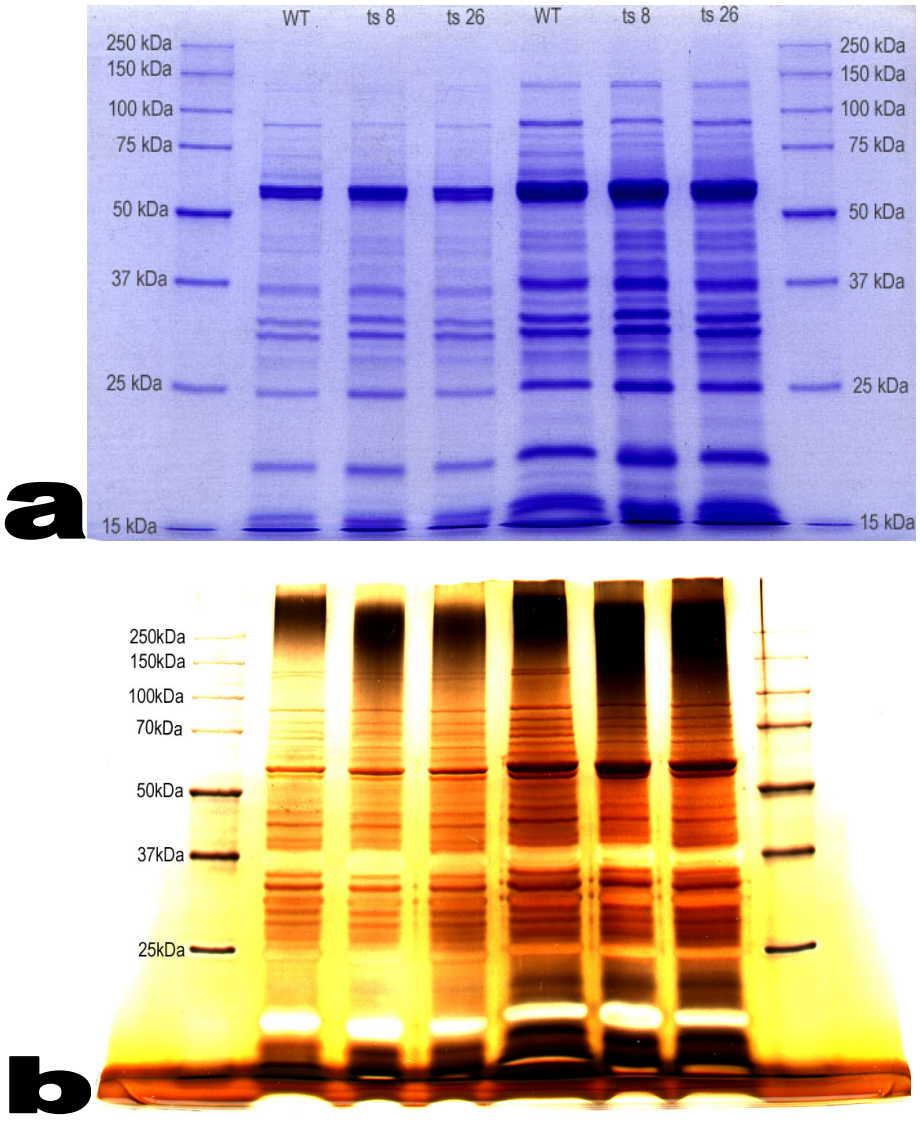

Figure 3.1. Stained SDS-PAGE gels of protein composition of purified particles. Viral particles grown at 40°C were purified by differential centrifugation, the protein composition of the particles was determined by electrophoresing particles by SDS-PAGE and staining. Coomassie stained gel (a) of purified wt40°C, Cts8 and Cts26 particles. Silver stained gel (b) of purified wt40°C, Cts8 and Cts26 particles. Lane 1 is a molecular weight marker, lanes 2 - 4 were loaded with 10 µl of sample, lanes 5 - 6 were loaded with 25 µl of sample.

Protein Composition of Mutants by Immunodetection

To further elucidate the protein composition of the mutant virions by Western blot, proteins from equivalent OD_{260} amounts of purified virions from Cts8, Cts26 and wt40ºC were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with a selection of different antibodies, representing an assortment of different enzymes and proteins found in the virus (Figure 3.2). The wt40ºC preparation of virions reacted with every antibody tested, as expected. Both of the mutant virion samples all tested positive with the antibodies. Of note, both mutant virion samples have a positive signal with the 4b protein antibody indicating that, even though their defect is in the A3L gene that encodes the 4b precursor, the mutant virions still have the 4b protein. The Cts26 virions were not tested with the A10 or D8 antibodies, but we speculate that since there was no difference in the Cts8 virion preparation when compared to wt40ºC virions, a significant difference with Cts26 is unlikely.

Viral Transcription in the A3L Mutants

To determine whether the aberrant particles were capable of transcription, purified virions were permeabilized and assayed for RNA synthesis (Gershowitz and Moss, 1979). Briefly, purified virions were added to a transcription mixture containing NP40, DTT, ATP, GTP and UTP, a limiting amount of CTP, and α^{32}P-CTP. RNAs produced after various times during the 37ºC incubation were precipitated in cold 5% TCA, collected on glass fiber filters and the radioactivity of the samples was counted in a liquid scintillation counter. The results, graphed in Figure 3.3, show that both wild type preparations, wt37ºC and wt40ºC, have increasing amounts of CMP incorporation throughout the 90 minute

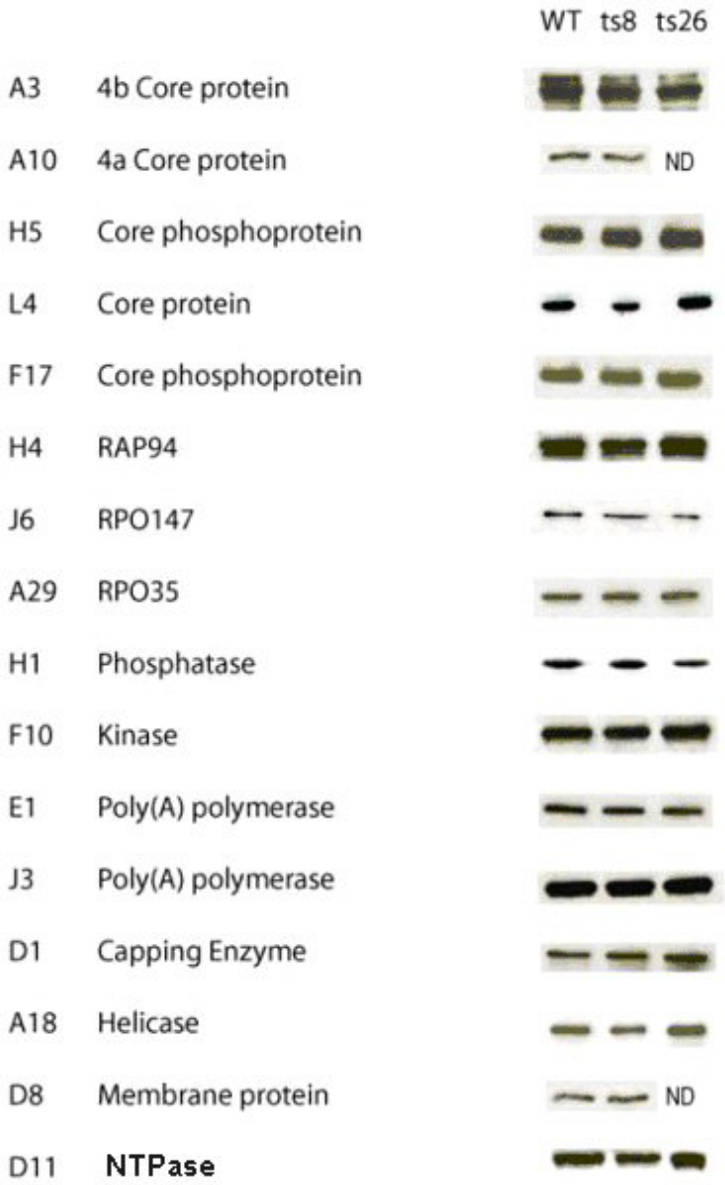

Figure 3.2. Western blot analysis of purified virions. Purified virions in Laemmli buffer were electrophoresed by SDS-PAGE and probed with antibody. The first column indicates the gene product detected by the antibody, the second column indicates the function of the gene product, and the last column shows the antibody signal as detected by enhanced chemiluminescence. ND indicates the experiment was not done.

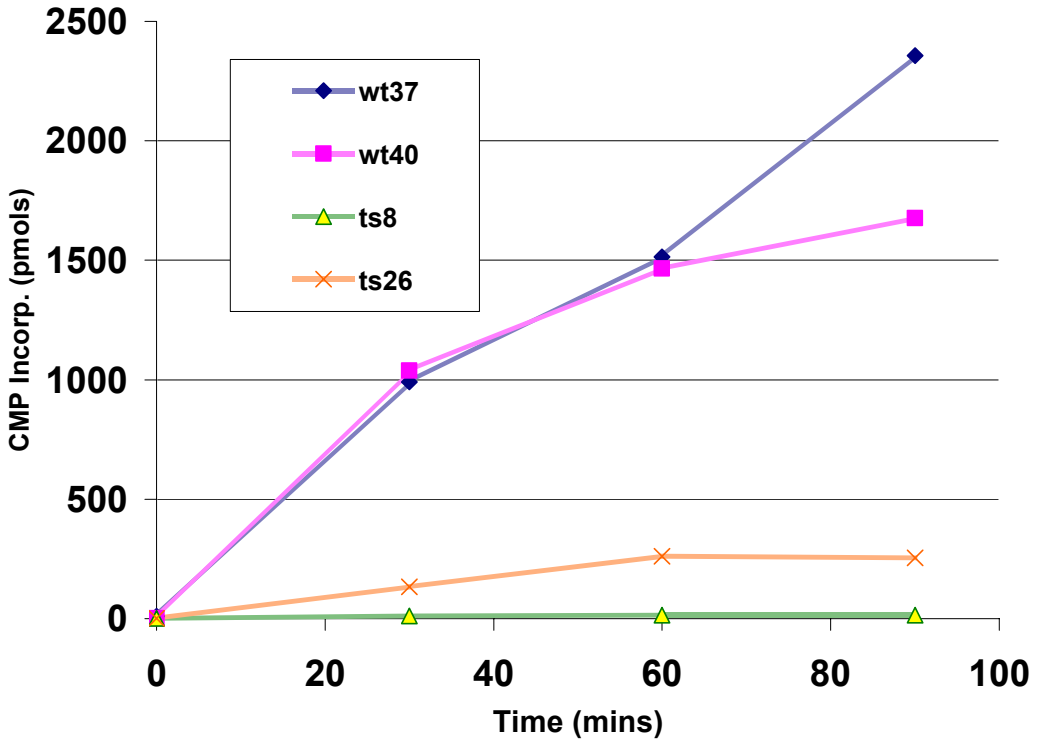

Figure 3.3. Transcription assay of purified virions. Virus particles grown at 40°C (or 37 °C in the case of wt37) were purified by differential centrifugation and assayed for transcription activity. 250 µl reactions were prepared containing 0.25 OD_{260} units of purified virus, 0.25 M Tris, 50 mM DTT, 50 mM $MgCl_2$, 25 mM ATP, 5 mM UTP, 5 mM GTP, 0.25 mM CTP, and 5 µCi α^{32}P-CTP. The reactions were incubated at 37°C and samples were drawn at various time points, precipitated in 5% TCA, and counted in a liquid scintillation counter. Counts were converted into picomoles of CMP incorporated and graphed on the Y-axis against time on the X-axis.

time point indicating that both wild type virion preparations are producing RNAs. Cts8 virions appear incapable of making RNA while Cts26 virions showed a slightly increased level of RNA production over Cts8, but were still five fold less effective than the wild type virions in RNA production. A second attempt with this protocol yielded the same results. This assay shows that, when grown at 40ºC, the mutant virions are nearly incapable of RNA synthesis.

To further examine the transcription abilities of the mutants, RNA polymerase and transcription factors were extracted from virus particles and assayed for transcription activity by Condit and co-workers (Kato et al., in preparation). The results showed that once isolated from the cores, the transcription enzymes of the mutant virions transcribe as well as wild type transcription enzymes (data not shown). This suggests that the A3L gene product is responsible for how the core and associated enzymes are organized and when mutated, as in Cts8 and Cts26, the resulting virions are unable to properly use their normal RNA polymerases.

E6R Mutant Viruses

Marker Rescue of E(2-8)a Mutants

Cts52 had been mapped to the E(2-8) region of the vaccinia genome previously by Condit and co-workers. To map the exact location of the mutation, the E(2-8) region was PCR amplified as a series of overlapping, 5 kb fragments and used in marker rescues to map the mutant viruses (Figure 3.4). Briefly, cells were infected with virus dilutions as determined by terminal dilution and transfected with a mixture of PCR amplified DNA and genomic mutant viral DNA. The infected cells were incubated at the non-permissive temperature for four days and stained with crystal violet to visualize plaques (Figures 3.5 - 3.7). The first of the marker rescue experiments showed that the mutants rescued with

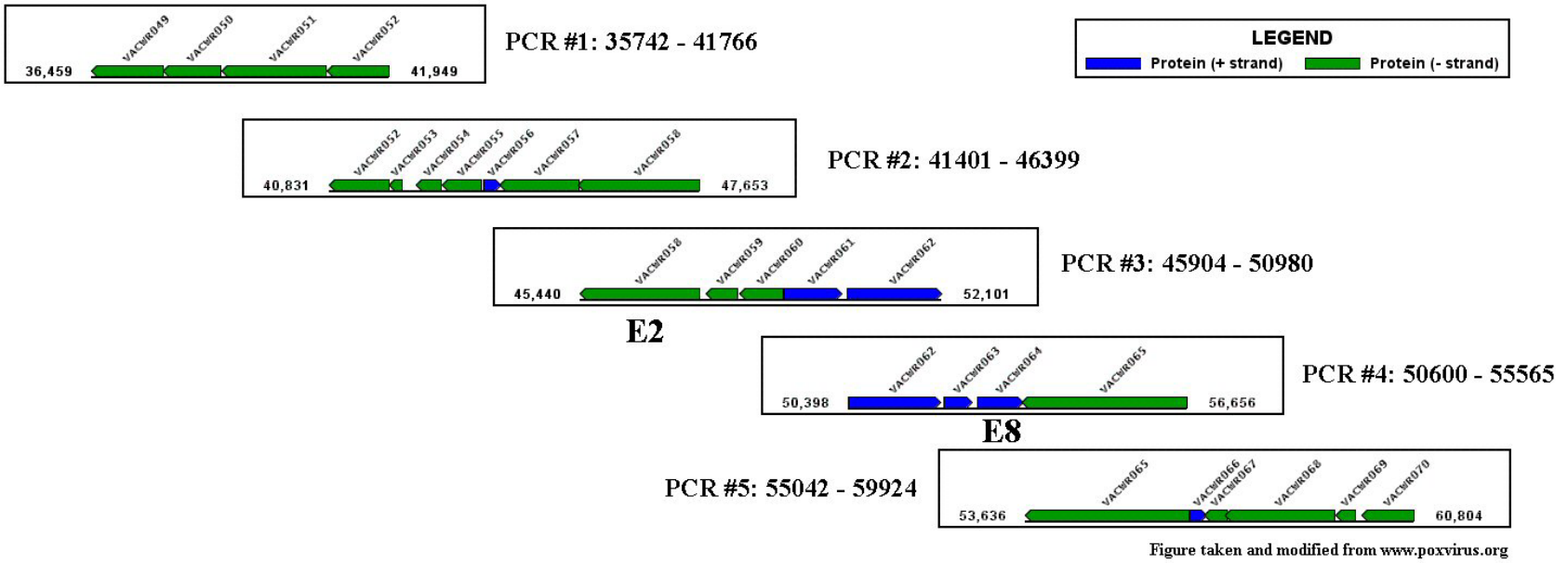

Figure 3.4. Overlapping 5 kb PCR products #1 – 5 of the vaccinia WR genome. 5 kb segments of vaccinia DNA were PCR amplified and used in marker rescue and sequencing experiments. PCR primers were designed and provided by Dr. Ben Luttge.

DNA from PCR #4. The genes from PCR #4 were then amplified with ORF specific primers yielding gene products for E6, E7 and E8. The PCR #4 product also included the E9 gene, but because all the mutants tested positive for DNA synthesis, the possibility of the mutation being located within E9, the DNA polymerase, was dismissed as improbable. Marker rescues were performed with the PCR amplified genes and all of the mutants rescued with DNA from the E6 gene. Signal was not as positive with PCR #4 as with the positive control or E6 because PCR #4 does not contain all of the E6 gene. PCR #4 contains all but the first 202 nucleotides of the E6 gene, which may explain the poorer rescue signal. If there are not enough nucleotides from the gene available for a successful recombination event, the rescue signal may be diminished. The ORF specific products indicate that the mutations in the three temperature sensitive viruses map to the E6R gene.

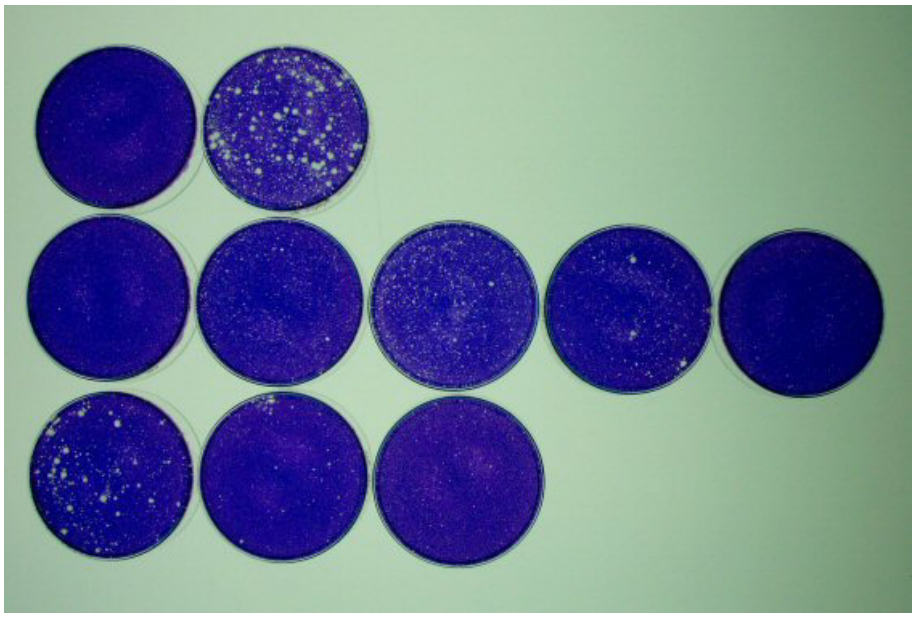

Figure 3.5. One-step marker rescue of Cts52. BSC40 monolayers were infected with a virus dilution determined from a terminal dilution experiment and incubated at 40°C. Inoculum was removed and cell were transfected with 1 µg of DNA fragment and 100 ng of mutant genomic DNA in a lipofectin solution. Infected cells were incubated for four days and stained with crystal violet.

From top left: untransfected negative control, transfected positive control using cosmid pWR18-53; second row, PCR products #1-5; third row, PCR products E6, E7 and E8.

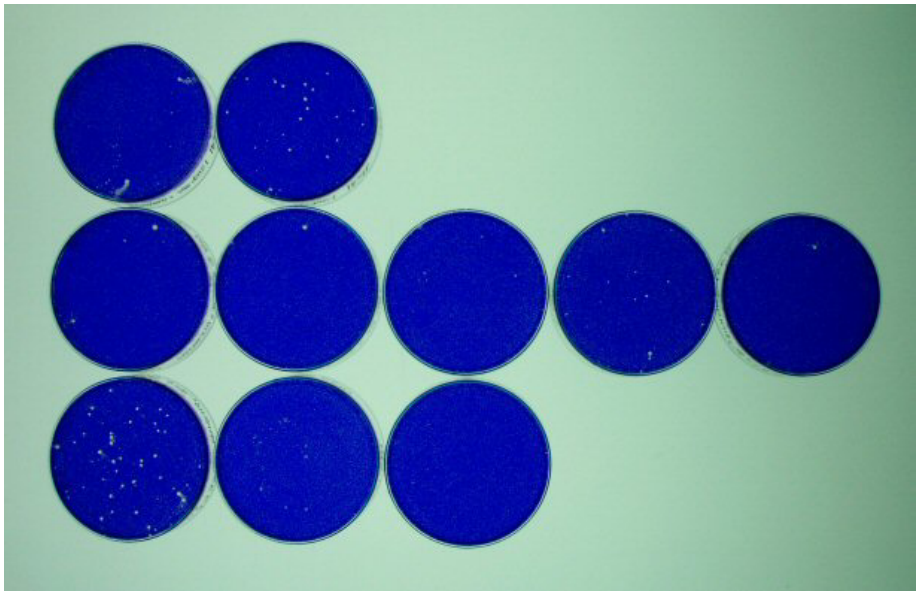

Figure 3.6. One-step marker rescue of Dts41. From top left: untransfected negative control, transfected positive control using cosmid pWR18-53; second row, PCR products #1-5; third row, PCR products E6, E7 and E8.

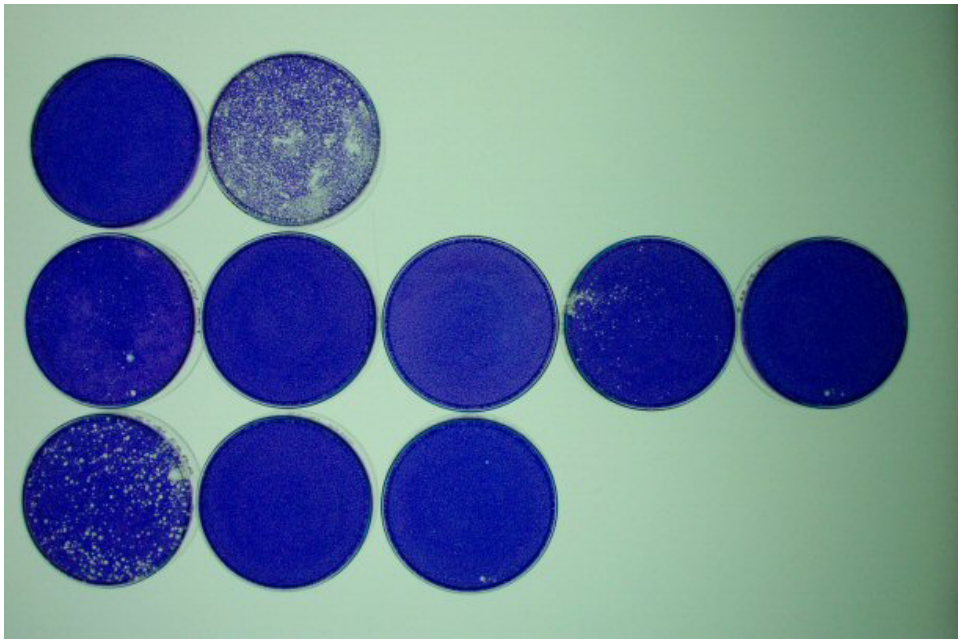

Figure 3.7. One-step marker rescue of Dts80. From top left: untransfected negative control, transfected positive control using cosmid pWR18-53; second row, PCR products #1-5; third row, PCR products E6, E7 and E8.

Sequence Analysis

To determine the exact nature of the mutations in the E6R genes of the mutant viruses a 3.341 kb region of DNA, containing the 1.7 kb E6 gene, was PCR amplified from each mutant and the two wild type strains, WR and IHD. This DNA was sequenced with several primers by the Sequencing Core at UF.

The data (Figure 3.8 and 3.9) show strain-specific changes when comparing the WR and IHD strains, six in all, only two of which result in amino acid changes: a threonine (T) to alanine (A) at codon 102, and a phenylalanine (F) to a glutamic acid (E) codon 124. The remaining strain-specific changes are silent. Cts52 has a missense mutation at nucleotide position 677 leading to a proline (P) to leucine (L) change; Dts41 had a missense mutation at nucleotide position 449 resulting in a proline (P) to leucine (L) change, and; Dts80 had two missense mutations at positions 756 and 778. The mutation at position 756 was silent, while the one at position 778 yields a leucine (L) instead of the expected phenylalanine (F).

Protein Synthesis and Processing of the E6R Mutants

To analyze the efficiency of protein synthesis in the mutant viruses in comparison with wild type, a pulse labeling reaction was performed. Briefly, confluent monolayers of BSC40 cells were infected with virus at an MOI of 10 for 30 minutes at either 31ºC or 40ºC. At various time points after infection, cells were pulsed with [^{35}S] methionine for 15 minutes and then harvested, electrophoresed by SDS-PAGE, and autoradiographed (Figure 3.10). The wild type virus at both temperatures shows a smear of proteins at the 0 hour time point. The proteins are a combination of viral and host cell proteins. Host protein synthesis is later shut down by the virus and by 6 HPI, the smear of host proteins

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WtWR  120 GTATCTAGTTAGCAATTTTCCTCAACATGTTATTACTAAGG 160
Cts52 120 GTATCTAGTTAGCAATTTTCCTCAACATGTTATTACTAAGG 160
WtIHD 120 GTATCTAGTTAGCAATTTTCCTCAACACGTTATTACTAAGG 160
Dts41 120 GTATCTAGTTAGCAATTTTCCTCAACACGTTATTACTAAGG 160
Dts80 120 GTATCTAGTTAGCAATTTTCCTCAACACGTTATTACTAAGG 160

WtWR  280 AAGAATTATTTATCGTTTAGTAACACAATACAATCGTACAA 320
Cts52 280 AAGAATTATTTATCGTTTAGTAACACAATACAATCGTACAA 320
WtIHD 280 AAGAATTATTTATCGTTTAGTAACGCAATACAATCGTACAA 320
Dts41 280 AAGAATTATTTATCGTTTAGTAACGCAATACAATCGTACAA 320
Dts80 280 AAGAATTATTTATCGTTTAGTAACGCAATACAATCGTACAA 320

WtWR  340 ACACAAGATGAAAAATTTTTAGAGGTTGCCAAATACATGGA 380
Cts52 340 ACACAAGATGAAAAATTTTTAGAGGTTGCCAAATACATGGA 380
WtIHD 340 ACACAAGATGAAAAATTTTTAGAGGTTGCCGAATACATGGA 380
Dts41 340 ACACAAGATGAAAAATTTTTAGAGGTTGCCGAATACATGGA 380
Dts80 340 ACACAAGATGAAAAATTTTTAGAGGTTGCCGAATACATGGA 380

WtWR  440 GAGGGGAACCCATCAAAGATATGGAAATCATTTTTTTAAAA 480
Cts52 440 GAGGGGAACCCATCAAAGATATGGAAATCATTTTTTTAAAA 480
WtIHD 440 GAGGGGAACCCATCAAAGATATGGAAATCATTTTTTTAAAA 480
Dts41 440 GAGGGGAACTCATCAAAGATATGGAAATCATTTTTTTAAAA 480
Dts80 440 GAGGGGAACCCATCAAAGATATGGAAATCATTTTTTTAAAA 480

WtWR  520 AGTGTTATAAGATTACTTATTTGGGCTTACCTAAGCAAGAA 560
Cts52 520 AGTGTTATAAGATTACTTATTTGGGCTTACCTAAGCAAGAA 560
WtIHD 520 AGTGTTATAAGATTACTTATTTGGGCATACCTAAGCAAGAA 560
Dts41 520 AGTGTTATAAGATTACTTATTTGGGCATACCTAAGCAAGAA 560
Dts80 520 AGTGTTATAAGATTACTTATTTGGGCATACCTAAGCAAGAA 560

WtWR  640 AGCAATCTAACAGAAACGTTTAGAGATTATATCTTTCCCGG 680
Cts52 640 AGCAATCTAACAGAAACGTTTAGAGATTATATCTTTCTCGG 680
WtIHD 640 AGCAATCTAACAGAAACGTTTAGAGATTATATCTTTCCCGG 680
Dts41 640 AGCAATCTAACAGAAACGTTTAGAGATTATATCTTTCCCGG 680
Dts80 640 AGCAATCTAACAGAAACGTTTAGAGATTATATCTTTCCCGG 680

WtWR  700 GTGTGGTTAAACGAAAGTATAGCTAATGATGCGGATATTGT 740
Cts52 700 GTGTGGTTAAACGAAAGTATAGCTAATGATGCGGATATTGT 740
WtIHD 700 GTGTGGTTAAACGAAAGTATAGCTAATGATGCGGATATCGT 740
Dts41 700 GTGTGGTTAAACGAAAGTATAGCTAATGATGCGGATATCGT 740
Dts80 700 GTGTGGTTAAACGAAAGTATAGCTAATGATGCGGATATCGT 740

WtWR  750 ACACGCCATTACCATGTATGATAAAATTCTTAGTTATATAT 790
Cts52 750 ACACGCCATTACCATGTATGATAAAATTCTTAGTTATATAT 790
WtIHD 750 ACACGCCATTACCATGTATGATAAAATTCTTAGTTATATAT 790
Dts41 750 ACACGCCATTACCATGTATGATAAAATTCTTAGTTATATAT 790
Dts80 750 ACACGCTATTACCATGTATGATAAAATTTTTAGTTATATAT 790

WtWR  1590 AAGGTTTTTAAGAGATAATCTATATCATGTAGAAGAATTCT 1630
Cts52 1590 AAGGTTTTTAAGAGATAATCTATATCATGTAGAAGAATTCT 1630
WtIHD 1590 AAGGTTTTTGAGAGATAATCTATATCATGTAGAAGAATTCT 1630
Dts41 1590 AAGGTTTTTGAGAGATAATCTATATCATGTAGAAGAATTCT 1630
Dts80 1590 AAGGTTTTTGAGAGATAATCTATATCATGTAGAAGAATTCT 1630
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Figure 3.8. Nucleotide alignment of wild type and mutant viruses. Green indicates the location of the corresponding mutant virus's mutation. Yellow indicates a polymorphism between the WR and IHD strains.

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WtWR            MDFIRRKYLIYTVENNIDFLKDDTLSKVNNFTLNHVLALKYLVSNFPQHVITKDVLANTN 60
Cts52           MDFIRRKYLIYTVENNIDFLKDDTLSKVNNFTLNHVLALKYLVSNFPQHVITKDVLANTN 60
WtIHD           MDFIRRKYLIYTVENNIDFLKDDTLSKVNNFTLNHVLALKYLVSNFPQHVITKDVLANTN 60
Dts41           MDFIRRKYLIYTVENNIDFLKDDTLSKVNNFTLNHVLALKYLVSNFPQHVITKDVLANTN 60
Dts80           MDFIRRKYLIYTVENNIDFLKDDTLSKVNNFTLNHVLALKYLVSNFPQHVITKDVLANTN 60

WtWR            FFVFIHMVRCCKVYEAVLRHAFDAPTLYVKALTKNYLSFSNTIQSYKETVHKLTQDEKFL 120
Cts52           FFVFIHMVRCCKVYEAVLRHAFDAPTLYVKALTKNYLSFSNTIQSYKETVHKLTQDEKFL 120
WtIHD           FFVFIHMVRCCKVYEAVLRHAFDAPTLYVKALTKNYLSFSNAIQSYKETVHKLTQDEKFL 120
Dts41           FFVFIHMVRCCKVYEAVLRHAFDAPTLYVKALTKNYLSFSNAIQSYKETVHKLTQDEKFL 120
Dts80           FFVFIHMVRCCKVYEAVLRHAFDAPTLYVKALTKNYLSFSNAIQSYKETVHKLTQDEKFL 120

WtWR            EVAKYMDELGELIGVNYDLVLNPLFHGGEPIKDMEIIFLKLFKKTDFKVVKKLSVIRLLI 180
Cts52           EVAKYMDELGELIGVNYDLVLNPLFHGGEPIKDMEIIFLKLFKKTDFKVVKKLSVIRLLI 180
WtIHD           EVAEYMDELGELIGVNYDLVLNPLFHGGEPIKDMEIIFLKLFKKTDFKVVKKLSVIRLLI 180
Dts41           EVAEYMDELGELIGVNYDLVLNPLFHGGELIKDMEIIFLKLFKKTDFKVVKKLSVIRLLI 180
Dts80           EVAEYMDELGELIGVNYDLVLNPLFHGGEPIKDMEIIFLKLFKKTDFKVVKKLSVIRLLI 180

WtWR            WAYLSKKDTGIEFADNDRQDIYTLFQQTGRIVHSNLTETFRDYIFPGDKTSYWVWLNESI 240
Cts52           WAYLSKKDTGIEFADNDRQDIYTLFQQTGRIVHSNLTETFRDYIFLGDKTSYWVWLNESI 240
WtIHD           WAYLSKKDTGIEFADNDRQDIYTLFQQTGRIVHSNLTETFRDYIFPGDKTSYWVWLNESI 240
Dts41           WAYLSKKDTGIEFADNDRQDIYTLFQQTGRIVHSNLTETFRDYIFPGDKTSYWVWLNESI 240
Dts80           WAYLSKKDTGIEFADNDRQDIYTLFQQTGRIVHSNLTETFRDYIFPGDKTSYWVWLNESI 240

WtWR            ANDADIVLNRHAITMYDKILSYIYSEIKQGRVNKNMLKLVYIFEPEKDIRELLLEIIYDI 300
Cts52           ANDADIVLNRHAITMYDKILSYIYSEIKQGRVNKNMLKLVYIFEPEKDIRELLLEIIYDI 300
WtIHD           ANDADIVLNRHAITMYDKILSYIYSEIKQGRVNKNMLKLVYIFEPEKDIRELLLEIIYDI 300
Dts41           ANDADIVLNRHAITMYDKILSYIYSEIKQGRVNKNMLKLVYIFEPEKDIRELLLEIIYDI 300
Dts80           ANDADIVLNRHAITMYDKIFSYIYSEIKQGRVNKNMLKLVYIFEPEKDIRELLLEIIYDI 300

WtWR            PGDILSIIDAKNDDWKKYFISFYKANFINGNTFISDRTFNEDLFRVVVQIDPEYFDNERI 360
Cts52           PGDILSIIDAKNDDWKKYFISFYKANFINGNTFISDRTFNEDLFRVVVQIDPEYFDNERI 360
WtIHD           PGDILSIIDAKNDDWKKYFISFYKANFINGNTFISDRTFNEDLFRVVVQIDPEYFDNERI 360
Dts41           PGDILSIIDAKNDDWKKYFISFYKANFINGNTFISDRTFNEDLFRVVVQIDPEYFDNERI 360
Dts80           PGDILSIIDAKNDDWKKYFISFYKANFINGNTFISDRTFNEDLFRVVVQIDPEYFDNERI 360

WtWR            MSLFSTSAADIKRFDELDINNSYISNIIYEVNDITLDTMDDMKKCQIFNEDTSYYVKEYN 420
Cts52           MSLFSTSAADIKRFDELDINNSYISNIIYEVNDITLDTMDDMKKCQIFNEDTSYYVKEYN 420
WtIHD           MSLFSTSAADIKRFDELDINNSYISNIIYEVNDITLDTMDDMKKCQIFNEDTSYYVKEYN 420
Dts41           MSLFSTSAADIKRFDELDINNSYISNIIYEVNDITLDTMDDMKKCQIFNEDTSYYVKEYN 420
Dts80           MSLFSTSAADIKRFDELDINNSYISNIIYEVNDITLDTMDDMKKCQIFNEDTSYYVKEYN 420

WtWR            TYLFLHESDPMVIENGILKKLSSIKSKSRRLNLFSKNILKYYLDGQLARLGLVLDDYKGD 480
Cts52           TYLFLHESDPMVIENGILKKLSSIKSKSRRLNLFSKNILKYYLDGQLARLGLVLDDYKGD 480
WtIHD           TYLFLHESDPMVIENGILKKLSSIKSKSRRLNLFSKNILKYYLDGQLARLGLVLDDYKGD 480
Dts41           TYLFLHESDPMVIENGILKKLSSIKSKSRRLNLFSKNILKYYLDGQLARLGLVLDDYKGD 480
Dts80           TYLFLHESDPMVIENGILKKLSSIKSKSRRLNLFSKNILKYYLDGQLARLGLVLDDYKGD 480

WtWR            LLVKMINHLKSVEDVSAFVRFSTDKNPSILPSLIKTILASYNISIIVLFQRFLRDNLYHV 540
Cts52           LLVKMINHLKSVEDVSAFVRFSTDKNPSILPSLIKTILASYNISIIVLFQRFLRDNLYHV 540
WtIHD           LLVKMINHLKSVEDVSAFVRFSTDKNPSILPSLIKTILASYNISIIVLFQRFLRDNLYHV 540
Dts41           LLVKMINHLKSVEDVSAFVRFSTDKNPSILPSLIKTILASYNISIIVLFQRFLRDNLYHV 540
Dts80           LLVKMINHLKSVEDVSAFVRFSTDKNPSILPSLIKTILASYNISIIVLFQRFLRDNLYHV 540

WtWR            EEFLDKSIHLTKTDKKYILQLIRHGRS 567
Cts52           EEFLDKSIHLTKTDKKYILQLIRHGRS 567
WtIHD           EEFLDKSIHLTKTDKKYILQLIRHGRS 567
Dts41           EEFLDKSIHLTKTDKKYILQLIRHGRS 567
Dts80           EEFLDKSIHLTKTDKKYILQLIRHGRS 567
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Figure 3.9. Amino acid alignment of wild type and temperature sensitive viruses. One letter amino acid codes are used, and mutations are shown in green and polymorphisms are shown in yellow.

is gone. At approximately 50 and 60 kDa, early viral proteins are detectable at 3 hours post infection and diminish as the infection continues. An intermediate protein, at approximately 35 kDa, is present at 3 hours post infection and is persistent through the remaining time points. Major core proteins, 4a and 4b, made late during infection, are detectable at 62 and 60 kDa, respectively, at 6 hours post infection. The Dts41 mutant virus shows no distinguishable differences from the wild type at either temperature. Similar results were obtained from Cts52 and Dts80 (data not shown). This supports the original data that classified Cts52 as having a normal protein synthesis phenotype.

In order to confirm that the protein processing of the mutant viruses was comparable to wild type virus, a pulse-chase experiment was performed. Cells were infected with virus and incubated for 8 hours then pulsed with [^{35}S] methionine and chased for various times. Samples were harvested, analyzed by SDS-PAGE and autoradiographed. The wild type results (Figure 3.11) show the normal protein processing phenotype. Protein bands present at 102 and 72 kDa in the pulse indicate the major core protein precursors, p4a and p4b. These proteins are processed, by the 8 hour time point, into the 60 and 62 kDa 4a and 4b, respectively. Proteolysis is evident in other areas as well. The mutant results show the same patterns of protein processing at the wild type. p4a and p4b bands, for example, are processed into the 60 and 62 kDa 4a and 4b proteins within the same time frames as the wild type. No changes in the protein processing by the mutant viruses are apparent, nor does the processing appear to be slowed in any of the mutant viruses even at the

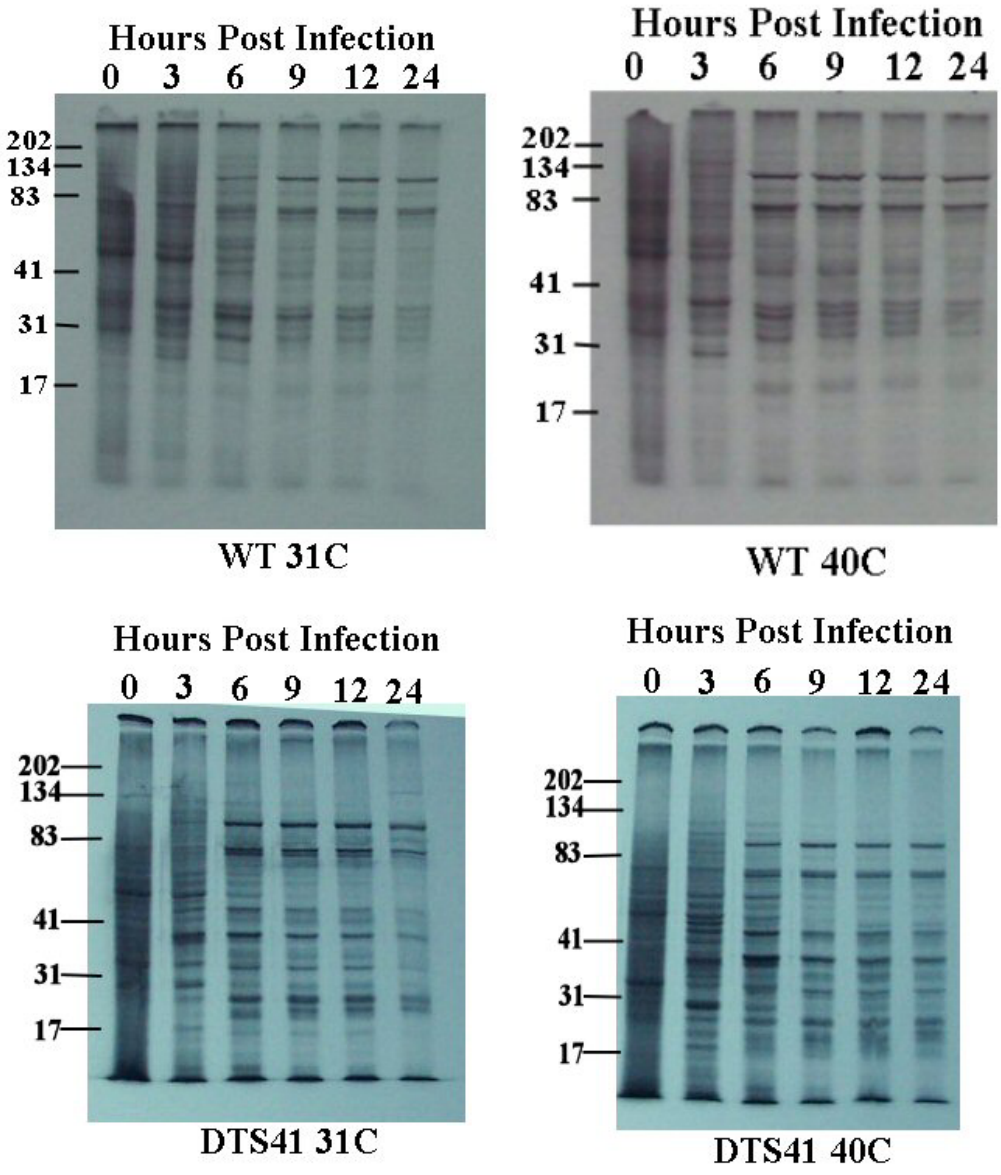

Figure 3.10. Protein pulse labeling experiment. BSC40 cells were infected with virus at an MOI of 10 and incubated for various times at either 31°C or 40°C. Cells were pulsed with radiolabeled methionine for 15 minutes, harvested and electrophoresed by SDS-PAGE. The above pictures are autoradiograms of the gels.

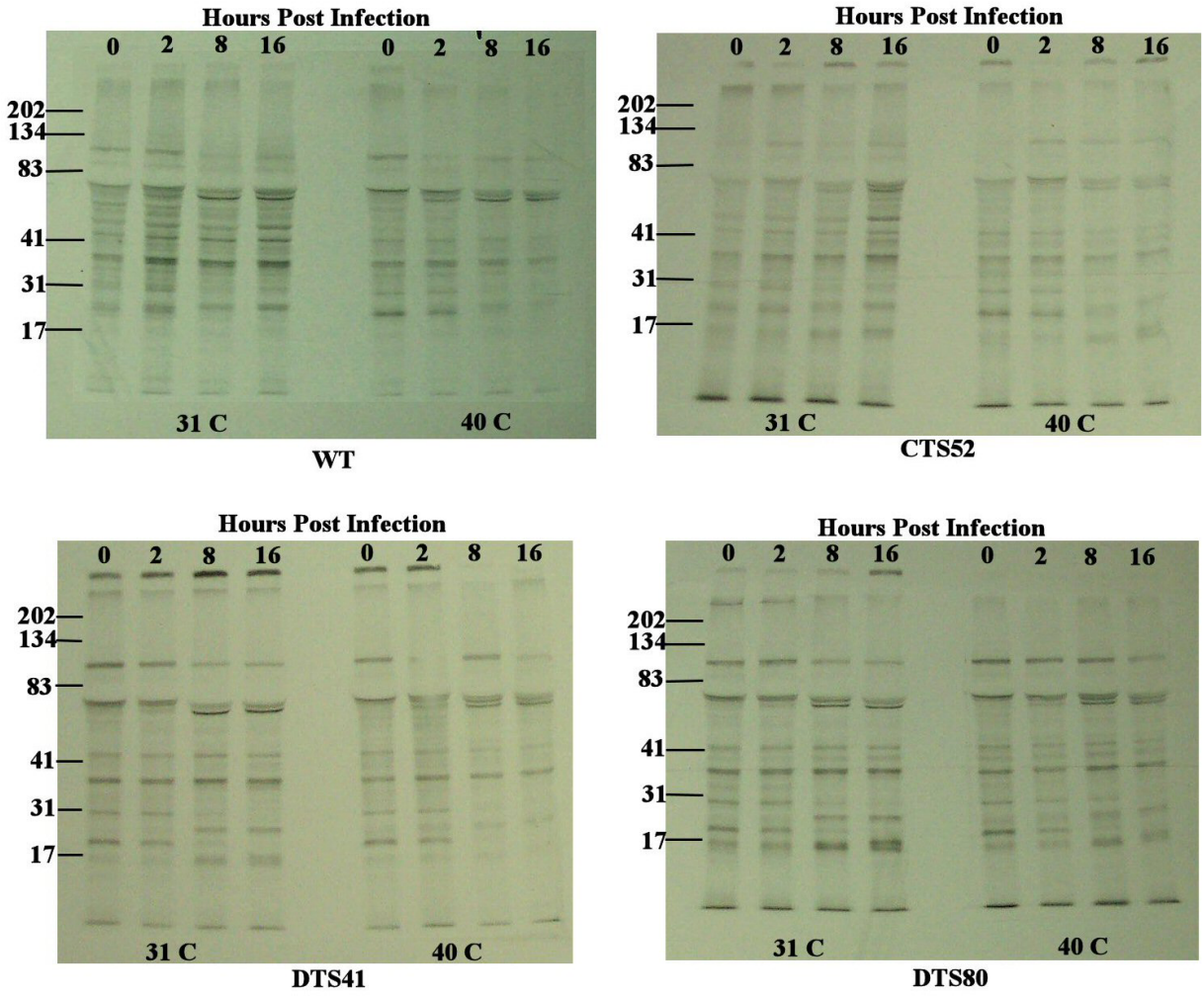

Figure 3.11. Protein pulse-chase experiment. BSC40 monolayers were infected at an MOI of 10 and incubated at either 31°C or 40°C for 8 hours. Cells were incubated with [^{35}S] methionine for 15 minutes (T=0) and replaced at either temperature for various times until cells were harvested, processed by SDS-PAGE, and autoradiographed. The 31°C samples are on the left and the 40°C samples are on the right of each gel.

non-permissive temperature. None of the mutant infections shows any difference from wild type in proteolytic processing by this assay.

Appearance of Virions Using EM

To determine how the mutations in the mutant viruses might affect morphogenesis, infected cells were examined by electron microscopy. Briefly, cells were infected with virus at an MOI of 10 and incubated at the non-permissive temperature for either 24 or 48 hours. Cells were fixed for EM and samples were processed and photographed at the EM Core at the University of Florida. The wild type infection (Figure 3.12) shows the normal stages of morphogenesis including crescents within the viroplasm (arrow 1), IVs and IVNs (arrow 2), and normal IMVs (arrow 3) located outside of the viroplasm. Results collected from the mutant virus infections (Figure 3.12) show that all three mutants have the same progression through morphogenesis and all stages of virion development, including IV, IVN, and IMV, and are indistinguishable from the wild type development. The EM pictures show that the E6R mutation does not affect the mutant virions with a visible structural change the way the A3L mutation does.

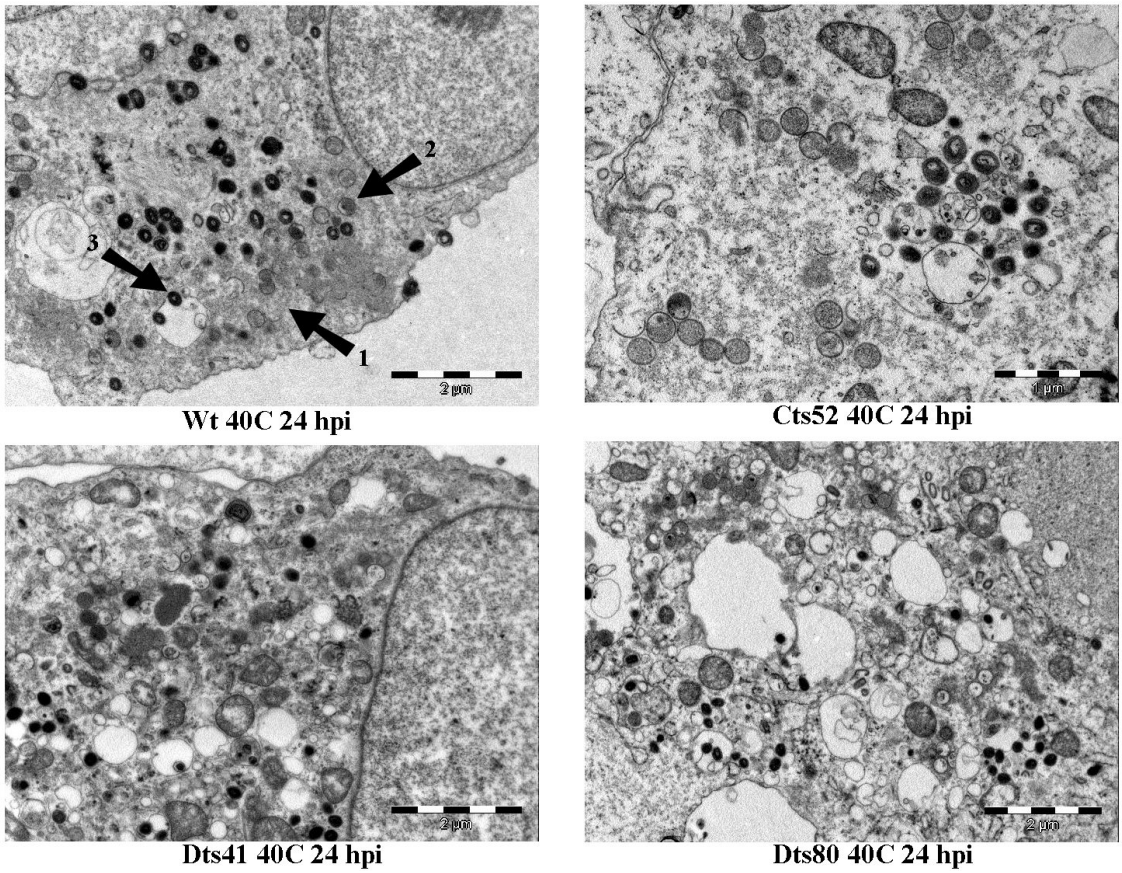

Figure 3.12. Electron microscopy of infected cells. BSC40 cells were infected with virus at an MOI of 10, incubated at the non-permissive temperature for 24 hours, and fixed for EM. Arrows in the wild type panel indicate viroplasm (arrow 1), IV with nucleiod (arrow 2), and IMV (arrow 3).

CHAPTER 4
DISCUSSION

The vaccinia genome encodes hundreds of genes with a wide range of functions. Using temperature sensitive vaccinia virus mutants, we can characterize the function of viral genes to help elucidate the mechanisms of viral reproduction. The "normal" DNA and protein phenotype mutants characterized in this study give us a better understanding of how viral genes affect the process of viral morphogenesis.

A3L

The goal of this work was to determine the nature and function of the gene responsible for the ts phenotype in Cts8 and Cts26. With the A3L mutant genotype information from marker rescue and sequencing experiments, and the mutant viruses' normal protein and DNA phenotypes, Cts8 and Cts26 were selected as candidates for morphogenesis research.

As an infection begins in a cell, vaccinia begins making protein products to further its replication. In a normal, wild type infection, these products are detectable within two hours of successful infection. Host cell protein synthesis is down-regulated and early viral proteins are made. The early viral mRNAs also encode for the DNA polymerase and more RNA polymerase for subsequent rounds of transcription. Late gene products aid in the process of viral assembly and include gene products that are packaged within the progeny virions. Experiments conducted by Kato et al. showed that Cts8 and Cts26 are identical to wild type in the synthesis of viral proteins, even at the non-permissive temperature. It was also shown that DNA synthesis in the mutant virus infections was

successful and that the DNA synthesized was resolved in a manner indistinguishable from wild type virus. With proof that the gene expression profiles and DNA synthesis patterns of the mutant viruses were identical to wild type, the role of the A3L gene in viral morphogenesis was investigated.

A normal virion must carry with it all the proteins, enzymes and other factors it needs to initiate a successful infection. In order to acquire all the proper factors, the virions must be built and packaged with the right complement of viral proteins. The composition of both wild type and mutant virions was tested by analysis of purified virions by SDS-PAGE, and after staining with Coomassie or silver, the wild type virion protein profile was seen (Figure 3.1). When compared side-by-side, the protein composition of the mutant virions did not appear any different from the wild type virions. The mutations in the A3L gene do not appear to affect the number or amount of proteins and enzymes the mutant virions carry. But while the virions appear intact in the how they are structured and in their composition, some element of their morphology is disrupted enough by the mutation to render them uninfectious. Purified virions were, therefore, tested for infectivity and mutant virions were 100 times less infectious than wild type virions.

The structural stages of vaccinia morphogenesis are easily seen by EM. The first of these stages, the appearance of the viroplasm, begins after the viral core uncoats and DNA replication begins. In a normal infection, the viroplasm is identifiable as a pool devoid of cellular organelles with a consistent texture. The cellular ER is in close proximity to the viroplasm and is the organelle responsible for supplying the material that becomes the viral crescents. Crescents eventually enclose an area of viroplasm to become

the spherical IVs and IVNs. Infections performed at the non-permissive temperature with the mutant viruses showed that the initial stages of viral morphogenesis proceed without incident and look like wild type infections. The structure and consistency of the mutant viruses' viroplasm was the same as wild type, as was the formation of crescents, IVs and IVNs.

Once formed, wild type viral IVN particles undergo a stage of maturation that changes their appearance from spherical shaped particles with electron dense nucleoids to brick shaped virions with dumbbell or brick shaped, electron dense cores. The new IMV particles are shuttled outside the viroplasm, wrapped by the cellular trans-Golgi complex, and transported to the plasma membrane for expulsion. The experiments with Cts8 and Cts26 at the non-permissive temperature showed that the transportation for the mutant derived particles appears to be the same as wild type, but morphological changes in the mutant virus particles are evident. Once the mutant IVNs are made, their similarity to wild type particles ends. The mutant particles do not mature into normal IMVs with the symmetry and structure of wild type IMVs. Under EM, mutant IMVs have an aberrant core structure. Mutant particles are asymmetric and have grossly disfigured cores that cannot be mistaken for wild type.

To more closely examine the elements packaged inside the aberrant cores, transcription experiments were performed. Because protein composition experiments determined that the mutant virions carry the same proteins as the wild type; the transcription experiments would help to determine whether the factors packaged in the virions were active. Little or no transcription from the mutant virions occurred. Cts26, while not entirely dead for transcription like Cts8, was still five fold less efficient for

transcription when compared to wild type. Since it was unknown whether the core enzymes were synthesized properly, an experiment was designed by Condit and co-workers to extract transcription factors from viral cores and assay those factors for transcription. The results of those experiments showed that the enzymes from within the mutant virions were perfectly capable of transcription, showing no differences when compared to wild type enzymes in contrast to their defect in particles.

In assimilating all the above results, it is evident that the mutant virions carry all the proteins that wild type virions carry and those proteins and enzymes appear fully functional when removed from the aberrant viral cores. It is therefore our conclusion that, because 4b is a major core protein and because all other elements of the virus particles are indistinguishable from wild type, the A3L mutation creates a defect in the organization of viral cores, and proper organization of the core and its factors is essential for a successful infection to occur. An interaction between 4b and another viral protein is possible, but given that 4b represents 11% of the virion mass and is supposed to be present in the outer core wall, we can speculate that any defects in such an abundant protein may be enough to disrupt the structure, organization, and function of the virion.

E6R

Cts52 was the first of the E(2-8)a ts mutants to be described as having a normal protein and DNA synthesis phenotype by Condit and co-workers. Dts41 and Dts80 were found to belong to the same complementation group by Lackner et al. As candidates for morphogenesis, the E(2-8)a group of ts mutants provided the opportunity to characterize viruses located in an area of the vaccinia genome that was relatively unexplored.

To discover which gene in the E(2-8) region was responsible for the ts mutations, it was necessary to first perform a marker rescue experiment and then to sequence the gene

that rescued the ts mutant viruses. During an infection at the non-permissive temperature of 40°C, a ts viral gene will produce a product that prevents the normal development of progeny virus particles. If there is a normal copy of the gene present in the ts infected cell, a recombination event can occur between the defective ts genome and the wt copy of the gene which results in a rebuilt wild type genome that is capable of growing at the non-permissive temperature. Several marker rescue experiments were performed which narrowed the range of possibilities from genes E(2-8) to genes E6 through E8 and finally to E6. The E6 genotype of each of the ts viruses was determined by sequencing the PCR amplified ts E6 genes. All the mutations were C to T changes, consistent with hydroxylamine mutagenesis, which altered the amino acid sequence of the mutant viruses. It is still unknown what effect the changes have upon the synthesis and possible processing of the E6 gene product.

To determine where the mutations disrupt the viral life cycle, it was necessary to examine all the steps in the life cycle cascade. The first step in the virus life cycle after entry is mRNA synthesis. Some gene products are only made during specific times during an infection, while other gene products are made throughout the infectious cycle. If a gene product is only synthesized at early times post infection, it can only be seen during the early time points in the protein pulse reaction and the same is true for intermediate and late gene products. The wild type protein synthesis profile (Figure 3.10) demonstrates some examples of early, intermediate and late gene products. There are early viral proteins present at approximately 50 and 60 kDa during the first two time points which disappear as the infection progresses. Intermediate protein signals appear after the first time points in the assay and can either stop being synthesized and disappear,

like those at 25 kDa, or can persist throughout the infection, like those at 35 kDa. Late proteins appear after intermediate gene products and persist through the remainder of the infection, like those at 102 kDa. The ts mutant virus' protein synthesis profiles were indistinguishable from the wild type profile indicating that the ts mutations do not affect the protein synthesis of the mutant viruses.

It was clear that the E6 mutation did not affect the synthesis of viral proteins during an infection, but it was necessary to determine if the mutation affected how those proteins were processed. Many viral proteins are synthesized in precursor forms that must be cleaved and processed to yield active forms the virus can use during its life cycle. Gene products produced by the virus can be involved in the proteolytic processing or can be those proteins which are processed. A protein pulse-chase labeling experiment helps to show if a ts mutation has any effect on the processing of any viral gene products. The wild type profile (Figure 3.11) shows the normal pattern of proteolytic processing. Proteins made by 8 hpi were radio labeled and incubated further to investigate the fate of the 8 hpi proteins. Many of the wild type proteins remain unchanged through the time course of the experiment, others are apparent at early time points and disappear later. The concurrent appearance of new protein bands in the autoradiograph demonstrates how a larger protein can be cleaved to yield smaller proteins that are used by the virus. None of the mutant viruses had any detectable differences from wild type protein processing in this assay leading us to the conclusion that the defective E6 gene product does not affect the protein processing of the viruses. While DNA synthesis was not tested in these experiments, Cts52's normal phenotype designation, given by Condit and co-workers in 1983, was presumed to apply to all the viruses in its complementation group.

Experiments to reaffirm this conclusion should be pursued in the future as should experiments to determine whether the E6 gene product affects the concatemeric resolution of DNA.

So while the ts mutants of the E6R complementation group appear normal for protein and DNA synthesis, the next elements of the vaccinia life cycle to examine for defects are morphogenesis and particle assembly. As described before, assembly of wild type virus particles begins in the viroplasm and progresses through maturation steps that leads to the eventual release of infectious EEVs or CEVs; these steps can be examined with EM of infected cells and purified viral particles. Examining the developing mutant particles under EM showed a normal formation of viroplasm in the cytoplasm of the host cell. The viroplasm was devoid of host cell organelles, just as in wild type. Crescents were present in the viroplasm in similar numbers as the wild type infection and did not appear malformed or defective. Immature mutant virions were normal in appearance and in the proper location, indistinguishable from wild type. EMs also show normal IMVs and even CEVs in the mutant infections leading to the conclusion that the mutation in E6R does not appear to involve the structural assembly of viral particles. Other ts mutant viruses, A28 for example, have been known to have this normal morphogenesis phenotype under EM only to be shown as defective for viropexis. These results show that more experiments must be performed on the E6R complementation group in order to elucidate the function of the gene. The nature of the protein product of E6R is unknown, as are any possible interactions that product may have with other viral proteins. And although they have tested positive for DNA, it remains to be seen if the mutant viruses are capable of resolving the concatemeric DNA. Purification of viral particles will allow

transcriptional and protein composition analyses to be performed as well as for characterization by EM.

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BIOGRAPHICAL SKETCH

Audra Strahl was born and raised in the San Francisco Bay Area. She attended the University of California at Berkeley where she earned her bachelor's degree in integrative biology. After moving to Florida in 1999, she married Brian Raisler and began her MS/MBA degree program at the University of Florida. With the two master's degrees she hopes to work in research management in the biotechnology and pharmaceutical industry.