MAPPING AND PHENOTYPIC CHARACTERIZATION OF TEMPERATURE SENSITIVE VACCINIA VIRUS MUTANTS Cts6 AND Cts9

By

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TABLE OF CONTENTS

LIST OF FIGURES

APPENDIX
KEY TO SYMBOLS

aa	amino acid
C-	carboxy
^{o}C	degrees centigrade
DNA	deoxyribonucleic acid
h	hour
kB	kilobase
kD	kilodalton
M	molar
µl	microliter
min	minute
mL	milliliter
mm	millimeter
mRNA	messenger ribonucleic acid
m.o.i.	multiplicity of infection
N-	amino
nm	nanometer
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid

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By

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Chair: Richard C. Condit
Major Department: Molecular Genetics and Microbiology

To further our understanding of morphogenesis in vaccinia virus, our laboratory is investigating several temperature sensitive mutants that display a "normal" phenotype. Normal mutants show no defects in viral DNA replication or protein synthesis, and thus are likely to be defective in virus assembly. In this report we describe two temperature sensitive mutants (Cts6 and Cts9) comprising a single complementation group.

Cts6 and Cts9 were mapped to the A28L gene by marker rescue. DNA sequence analysis of the mutant A28L gene reveals a C-terminal frameshift mutation in both mutants, predicting that both encode C-terminal truncated proteins. Western blot analysis with A28L specific polyclonal antibody shows that the A28L protein is contained in virions. Metabolic labeling of proteins in infected cells, and pulse chase analysis of labeled proteins, shows that viral protein synthesis and proteolytic processing of virion precursor proteins in Cts6 and Cts9 infected cells is indistinguishable from a wild type virus infection at the nonpermissive temperature. Electron microscopic analysis of virus

infected cells shows that wild type and mutant virus infections were indistinguishable at both the permissive and nonpermissive temperatures. In an in vitro transcription assay purified mutant particles produced at the nonpermissive temperature were able to transcribe at the same level as wild type. However the purified particles at the nonpermissive temperature were not infectious at the permissive temperature, suggesting a defect in cell entry. We hope that continued study of Cts6 and Cts9 will lead to a further understanding of vaccinia virus cell entry.

CHAPTER 1
INTRODUCTION

General Vaccinia Background

Vaccinia virus is a member of the *Poxviridae* family. It contains a large, double-stranded DNA genome, and replicates solely in the cytoplasm of vertebrate cells. Vaccinia virus is known mostly for its use in the eradication of smallpox, caused by another member of the *Poxviridae* family, variola virus. The vaccinia genome (192 kB) encodes over 200 polypeptides, half of which are incorporated into the virion. Because poxviruses carry out replication and transcription of a DNA genome in the cytoplasm, it is an ideal model system for studying mRNA synthesis and processing. Vaccinia virions have a smooth, rounded rectangular appearance, and measure about 350 x 270 nm under cryoelectron microscopy. The intracellular mature virion (IMV) is the most abundant infectious particle, composed of a dumbbell-shaped core, surrounded by a lipoprotein bilayer. The core contains the viral genome, enzymes required for transcription and replication in the cytoplasm, and additional proteins thought to have structural roles (Moss, 2001). This unique structure of vaccinia virus is assembled during an intricate morphogenesis process that is the focus of this study.

Virus Life Cycle

Virion Structure and Entry

Although IMV is the most abundant form of the virus, three others are produced during the life cycle. Intracellular mature virions are well suited to mediate transport among hosts, but are not well suited for spreading the virus within the host because of its

vulnerability to complement and antibody. Other forms that exist are intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV), and extracellular enveloped virus (EEV) (Smith et al., 2002). These different infectious forms of the virus, combined with the fact that cellular receptors and viral attachment proteins have not been identified, complicate studies of virus entry into the cell. Although IMVs are commonly used in the laboratory to infect cells, they are only released after cell lysis. The IMVs are thought to enter the cell by fusion with the plasma membrane or vesicles. The EEVs and CEVs are more important in cell-to-cell spread. The EEV is thought to enter by a mechanism that involves endocytosis followed by low-pH disruption of the EEV outer membrane and then fusion of the released IMV with endosomal membrane. Only four IMV proteins have been implicated in cell entry and penetration: L1R, A27L, D8R, and H3L. Difficulties with studying EEV entry led to only identifying two EEV proteins: A34R, B5R (Moss, 2001). The mechanisms of vaccinia cell entry and penetration are still under investigation.

Figure 1 is an overview of the vaccinia virus life cycle. It is generally accepted that the final result of virus entry is the delivery of the core into the cytoplasm of the cell. It has been shown that the uncoating of viral cores is blocked by prevention of transcription or translation, suggesting the requirement for a virus-induced or virus-encoded protein. A putative 23-kD protein with trypsin-like activity was partially purified from infected cells and is thought to have a role in uncoating of the cores. Before the complete uncoating of the virus core takes place, early genes are transcribed, including DNA polymerase, RNA polymerase, growth factors, immune defense molecules, and intermediate transcription

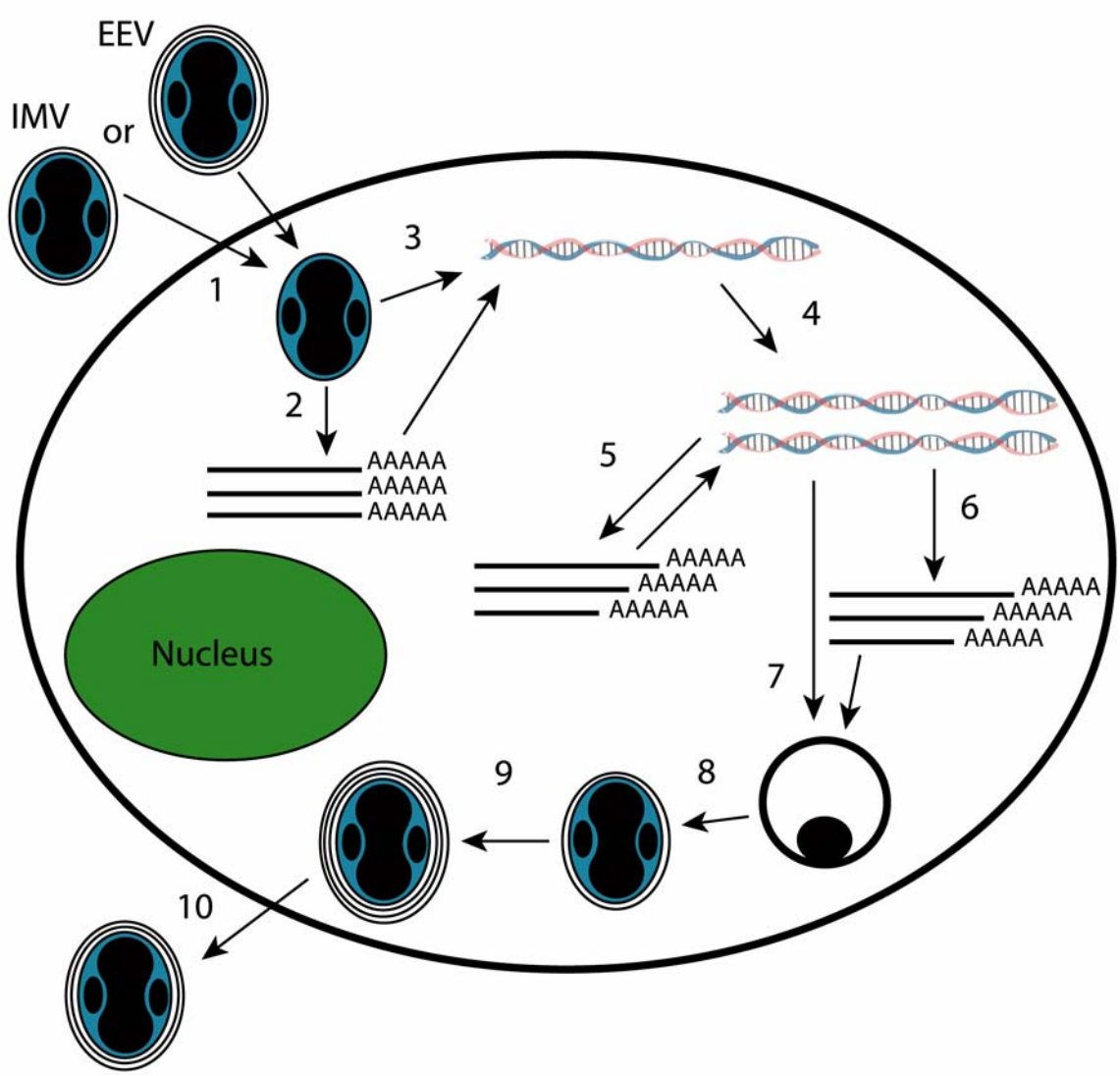


Figure 1. Vaccinia virus life cycle. One of the infectious forms of virus attaches to the cell, penetrates the membrane, and releases the core into the cytoplasm (1). The cores synthesize early mRNAs that are translated into a variety of proteins including intermediate transcription factors and DNA replication factors (2). The virion uncoats (3), the DNA genome is replicated (4), and intermediate genes are expressed (5). Intermediate genes encode late transcription factors, allowing for late gene transcription (6). Late genes encode early transcription factors, enzymes, and virion structural proteins. Newly assembled virions (7) undergo maturation (8) to form intracellular mature virions. These virions are wrapped by modified Golgi membranes (9) and transported to the periphery of the cell. Fusion of the outer membrane with the plasma membrane results in the release of extracellular enveloped virions (10).

factors (Moss, 2001). Because enzymes that carry out early gene transcription and modification of early RNAs are packaged in virions, it is possible to perform an *in vitro* transcription reaction with purified virus particles. The purified virus particles can be permeabilized with neutral detergent, incubated with nucleoside triphosphates, and they will produce authentic fully modified early mRNA (Condit et al., 2002).

Early Gene Expression

During a vaccinia infection, gene expression is regulated at the level of transcription. There are three classes of gene expression: early, intermediate, and late. Each gene class has its own distinct promoters and cognate *trans*-acting factors, mostly virus-coded. The regulation of vaccinia genes can be thought of as a "cascade" because the factors required for initiation of each gene class are encoded primarily by the genes of the preceding class. A virus-coded, multi-subunit, RNA polymerase carries out the RNA synthesis. This RNA polymerase is packaged into the virion late during the infection, and is synthesized throughout the infection. The RNA polymerase exists in two forms: one is specific for early genes, and the other is specific for intermediate and late genes. These two forms of RNA polymerase have eight subunits in common, ranging from 7 kD to 147 kD. The early gene-specific form has an additional 94 kD subunit, which is required for recognition of early promoters (Condit et al., 2002).

DNA Replication

As previously discussed, virus uncoating takes place after early genes are transcribed. Many early gene products are factors required for replication of the viral genome. One of the most unique aspects of poxviruses is that DNA replication occurs entirely in the cytoplasm of the infected cell, a characteristic that is only shared with African swine fever virus. A discrete cytoplasmic area of replication free of cellular

organelles (termed factory areas, or virus factories) has been located using light and electron microscopy. Some of the important virus-encoded DNA replication proteins are the DNA polymerase, nucleoside triphosphatase, DNA glycosylase, B1R protein kinase, and DNA ligase. DNA replication begins 1 to 2 hours after infection, and results in about 10,000 copies of the genome, about half of which are packaged into virions. Vaccinia virus contains a unique genome structure: a double stranded genome with covalently closed inverted terminal repetitions. Currently there are still large gaps in the understanding of its DNA replication. The accepted model for replication proposes that vaccinia replicates through concatemer intermediates, with the hairpin termini having an essential role. Replication of the genome then allows for the transcription of intermediate and late genes (Moss, 2001).

Postreplicative Gene Expression

Inhibitors of DNA replication prevent the transcription of intermediate and late genes; thus these genes are referred to as postreplicative. As discussed earlier, the RNA polymerase that is responsible for intermediate and late genes contains eight of the nine subunits that are present in the early RNA polymerase. Transcription of intermediate genes requires five early gene products termed Vaccinia Intermediate Transcription Factors. Intermediate genes encode proteins that are involved in immune defense, virus morphogenesis, and late transcription. Late genes require four viral intermediate gene products called Vaccinia Late Transcription Factors. Late genes also require one or more host proteins. The products of late genes include RNA polymerase, immune defense proteins, early gene transcription factors, poly(A) polymerase, and virion morphogenesis proteins. Intermediate and late gene mRNAs are thought to be capped and polyadenylated by the same enzymes responsible for modifying early mRNAs (Condit et al., 2002). After

the late genes are expressed and the DNA concatemers have been resolved, assembly of new virus particles begins (discussed in detail in the next section).

Morphogenesis

Investigations of morphogenesis using electron microscopy have reported that the formation of new virions takes place in the virus factories that were discussed earlier. Figure 2 is an overview of vaccinia morphogenesis. The first visible structures to appear are called crescents, which are composed of lipid and virus coded protein (Smith et al., 2002). There is ongoing debate about the origins of the crescents. Some believe that crescents are synthesized *de novo* and that they have no apparent continuity with cellular organelles (Dales et al., 1968;Grimley et al., 1970). Others believe that the crescents are a pair of tightly apposed membranes that are derived from cisternae originating from the intermediate compartment between the endoplasmic reticulum and the Golgi complex (Sodeik et al., 2002). The *de novo* model for membrane synthesis contradicts the principle that membranes grow from existing membranes. In contrast, the double membrane theory fits what we do know about membrane biosynthesis (Smith et al., 2002). Crescents continue to mature in spherical structures called immature virions (IV) and are filled with a matrix that exists in the *virus factory*. The crescents contain late viral membrane proteins, while the electron dense matrix enclosed in them contains viral core proteins. The electron dense matrix appears to condense, viral DNA is packed into the IV and DNA nucleoids appear. Particles with nucleoids are termed immature virions with nucleoid (IVN). The packing of DNA into the IV does require several virus-encoded proteins. The next step is the maturation of the IVN into IMV, which is a process that coincides with the proteolytical cleavage of core proteins such as 4a, 4b, and p25.

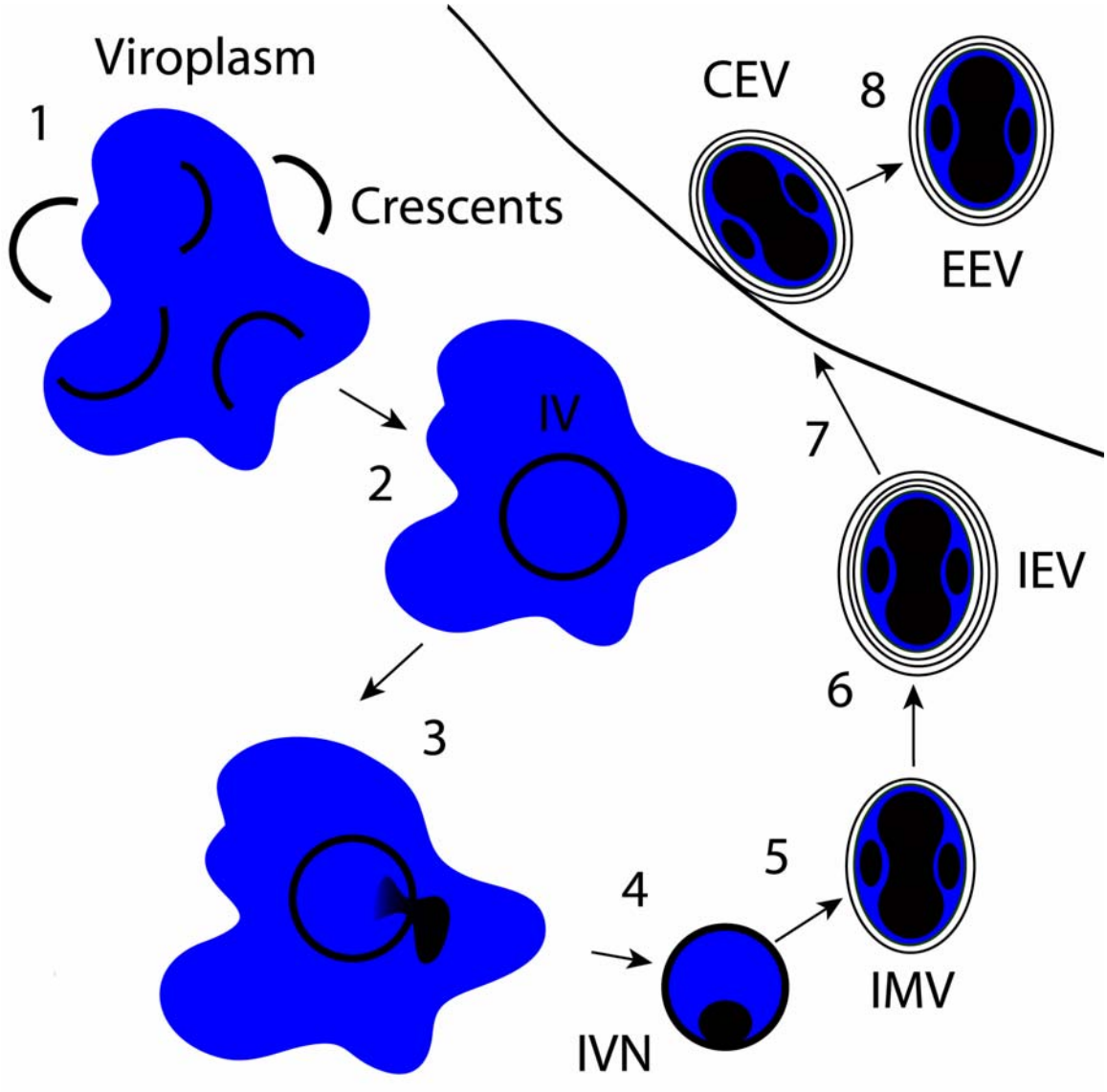


Figure 2. Vaccinia virus morphogenesis begins with the appearance of the viroplasm or virus factory followed by the emergence of crescents (1). The crescents mature and into IVs (2) and are packed with DNA (3). The DNA and viral matrix inside the IV condense to form IVN (4). The IVN then undergo a morphogenesis into IMV (5). The IMV are then rapped with two additional membranes to form IEV (6). The IEV move to the periphery of the cell and the outer membranes fuses with the cell membrane exposing the CEV on the cell surface (7). The CEV may be released from the cell as EEV (8).

The cleavage of these proteins triggers the transition from the spherical IVN to the brick-shaped Intracellular mature virus (IMV), and the generation of the distinct core structure of the IMV (Sodeik et al., 2002).

After IMV formation the particles move to sites where they are wrapped in a double layer of membrane to form an intracellular enveloped virus (IEV). The movement of the IMV from the virus factory to where they are wrapped in additional membranes this is thought to be dependent upon A27 protein and microtubules. Some of the IMV are not wrapped in additional membranes and stay in the cell until cell lysis. At later times during infection a larger portion of the IMV remain unwrapped, possibly due to the depletion of wrapping membranes. The IEV form of the virus is an intermediate between the IMV and the cell-associated enveloped virus (CEV). The IEV form functions to transfer the virus to the cell periphery with the help of the F12 protein, and to release the virus particle from the cell with a membrane that shields the sensitive IMV particle from antibody and complement (Smith et al., 2002).

Once the IEV reaches the cell surface the outer envelope fuses with the cell membrane allowing the CEV to be exposed on the cell surface. The A36 protein has been shown to be present in the IEV and not in the CEV. A36 protein was shown to be concentrated on the cytosolic face of the plasma membrane beneath CEV and is thought to aid in the emersion of CEV on the cell surface by the formation of actin tails (van Eijl et al., 2000). Along with the A36 protein the F12, A33, A34, and B5R proteins are all involved in actin tail formation. Actin tails are able to grow considerable distances from the cell surface and aid in virus penetration into surrounding cells. As actin tails grow longer they may detach from the cell with the CEV still at its tip, alternatively the CEV

may be released to give Extracellular enveloped virus (EEV). Both the EEV and the CEV form of the virus contain 3 membranes surrounding the core. A33, A34, A36, and B5R proteins are also involved in the release of the EEV. Although EEV represents only a small fraction of virus infectivity, it is biologically very important for long-range virus spread. Not only does the EEV form have a higher infectivity than other forms, but also it is resistant to complement and neutralization by antibody.

Temperature sensitive (ts) vaccinia virus mutants have been an integral part to understanding viral morphogenesis. Many ts mutants that have defects in different stages of morphogenesis have been isolated and described. A ts mutation in the H5 gene produces a virus that has "curdled virosomes" and does not produce any of the structures of morphogenesis (Demasi et al., 2000). The F10 protein has been implicated in the formation of viral crescents through the use of a ts mutant (Wang et al., 1995). A ts mutant that contains a J1 defect is able to produce crescents, however, the mutant forms aberrant, empty IVs (Chiu et al., 2002). A ts mutant with a mutation in the I8 gene produces non-infectious IMVs (Fathi et al., 1991).

The Problem

A collection of 65 temperature sensitive vaccinia virus mutants have been isolated and subsequently described (Condit et al., 1981;Condit et al., 1983). The goal of creating this collection was to have a thorough, systematic genetic analysis of vaccinia virus genes. Complementation analysis was performed on all 65 mutants, showing that these mutants comprise 32 complementation groups. These complementation groups were then analyzed to see if they were able to synthesize DNA and proteins at the nonpermissive temperature. The results of these tests produced four phenotypes: (1) normal, which contains mutants that were able to synthesize DNA and early and late proteins at the

same level as wild type virus. (2) DNA-negative are mutants that cannot synthesize DNA and are only able to synthesize early proteins. (3) Defective late mutants display normal DNA, and early protein synthesis, but late protein synthesis is either weak, delayed, or both. (4) Abortive late mutants are also able to synthesize DNA and early proteins, however, their late protein synthesis begins and later aborts. Within this collection of mutants is a complementation group that contains Cts6 and Cts9, which display wild type DNA and protein synthesis, and thus were placed in the normal category (Condit et al., 1983). Mutants that are classified as "normal" usually are defective in morphogenesis. Cts6 was mapped by marker rescue, using a cosmid clone library, to the right half of the A fragment of the HindIII restriction map (Thompson et al., 1986). The purpose of this research project is to continue the preliminary characterization of this complementation group to obtain a better understanding of viral morphogenesis. This includes the genetic mapping of the mutants to a single open reading frame and sequencing this gene to determine the nature of the mutations. A phenotypic characterization will also be described which includes protein synthesis and processing, virion transcription and infectivity, and protein detection in infected cells and purified virions. Electron microscopy analysis of the mutants is also examined in order to get a detailed look at their morphogenesis.

CHAPTER 2
MATERIALS AND METHODS

Cells and Viruses

The BSC40 cells and wild type vaccinia virus strain WR, Cts6 and Cts9, the methods for cell and virus culture, virus infection, and plaque titration have been previously described (Condit et al., 1981;Condit et al., 1983). The non-permissive temperature for mutant infections was maintained between 39.5^{o}C and 40^{o}C, which will be labeled in figures as 40^{o}C for convenience.

Marker Rescue

Marker rescue was performed as previously described (Meis et al., 1991; Thompson et al., 1986). The cosmid clones used for the initial mapping and later as a positive control have been previously characterized (Thompson et al., 1986). B. Luttge and R. Moyer generously supplied PCR primer sets also used for the initial mapping and as positive and negative controls. Alternatively, PCR products used in the rescue were generated using primers that specifically amplified A27L, A28L, and A29L open reading frames (ORFs). PCR products were also generated using primers that specifically amplified A25L-A26.4L, A25L-A27L, A27L-A28L, A27L-A29L, and A28L-A29L ORFs. Integrated DNA Technologies, Inc. (IDT Coralville, IA) synthesized all oligonucleotides used as primers.

DNA Sequence Analysis

DNA sequence of the A27L and A28L genes from wt, Cts6, and Cts9 viruses was obtained by direct sequencing of PCR products amplified from total infected cell DNA.

Total infected cell DNA was isolated using the Qiagen Dneasy miniprep spin columns (Qiagen) according to the manufacturer's instructions for isolation from cells in culture as previously described (Latner et al., 2000). Plasmids pT-Adv-A28L and pET-16b-A28L construction is described below. Their isolation from *Escherichia coli* culture was done using the Sigma GenElute High Performance Plasmid Maxiprep Kit (Sigma) following the manufacturer's instructions for vacuum format. All DNA mentioned above was sequenced by the University of Florida ICBR DNA Sequencing Core Laboratory.

Electron Microscopy

Confluent monolayers of BSC40 cells on 60 mm dishes were infected with wt or mutant viruses at an m.o.i. of 10. The cells were incubated at either 31 ^{o}C or 40 ^{o}C for 24, 36, or 48 h. At the appropriate time postinfection the cells were washed with 4 mL of 0.1 M sodium cacodylate buffer, pH 7.4, then 2 mL of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer was added to each dish, the dishes were then incubated at room temperature for 1 hour with occasional rocking. The cells were then scraped from the dishes, pelleted at 700 g for 5 min, resuspended in 0.1 M sodium cacodylate buffer, and stored at 4 ^{o}C until further processing. The cells were collected by centrifugation, post-fixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol solutions and 100% acetone, and embedded in Embed-812 resin mix. En bloc staining was done using uranyl acetate solution in 75% ethanol. After thin sectioning, the samples were post stained in uranyl acetate and Reynolds lead acetate. After mounting the samples were viewed under a Hitachi H7000 transmission electron microscope. Fixating, embedding, thin sectioning, staining, and microscopy were done with the assistance of the University of Florida ICBR Electron Microscopy Core Laboratory.

Recombinant DNA Clones

The PET-16b-A28 contains the A28L open reading frame and was created as follows. The A28L gene was PCR amplified from vaccinia WR DNA using primers that add a NdeI site at the 5' end and a BamHI site at the 3' end. This PCR product was then ligated into the 3' T overhangs of the pT-Adv vector (Clontech) making pT-Adv-A28L. pT-Adv-A28L was then digested with NdeI and BamHI, the fragment containing A28L was isolated and cloned into NdeI-BamHI cut pET-16b (Novagen) making pET-16b-A28L.

Antibodies

The pET-16b-A28L was used for the bacterial overexpression of polyhistidine-tagged A28 according to the pET system manual (Novagen). Briefly, Rosetta(DE3)pLysS containing pET-16b-A28L were grown in 50 mL LB containing 34 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37ºC overnight. We used 25 mL of the preculture to inoculate a 500 mL culture that was grown at 37ºC. After the OD_{590} of the culture reached 0.6, the culture was induced by the addition of IPTG (0.1M stock) to a final concentration of 1mM and incubated for 4 hours at 37ºC. Bacterial cells were sedimented, and the pellets were resuspended in 20 mL BugBuster Protein Extraction Reagent (Novagen) and incubated at room temperature for 10 min. After freezing and thawing the sample was treated with 125 units of RQ1 RNase-Free DNase (Promega). The inclusion bodies were then isolated and washed according to Antibodies (Harlow et al., 1988). The inclusion body preparation was then sent to Strategic Biosolutions for polyclonal antibody production using the standard two rabbit 70-day protocol. This antibody will be referred to as the anti-protein antibody for convenience. An anti-peptide antibody was also created by Sigma Genosys. This antibody was made from the peptide

DRRVQDVNDTISDVKQKWRC, which spans amino acids 56-75 of A28. This antibody will be referred to as the anti-peptide antibody for convenience.

Purification of Virus Particles

Confluent monolayers of BCS40 cells on 150 mm dishes were infected with wt, Cts6, or Cts9 viruses at an m.o.i. of 10 at 40^{o}C. 24 h after infection cells were harvested and purification was performed by sedimentation through preformed discontinuous sucrose density gradients as previously described (Joklik, 1962b). Purified virions were quantified by optical density at 260 nm (1 OD_{260} = 64 μg virus). Infectivity of the purified virus was assayed by plaque titration on BSC40 cells at 31 ^{o}C or 40 ^{o}C.

Western Blots

After separation of proteins on 12% SDS-PAGE or 10-20% linear gradient precast (Bio-Rad) gels, the proteins were transferred to nitrocellulose membrane (Bio-Rad) at 100V for 1hr. The blots were then blocked overnight in 5% nonfat dry milk dissolved in 0.05 M Tris, pH 7.5; 0.15 M NaCl; 0.1% Tween-20 (TBST). The blots were washed twice in TBST for 5 min and then re-blocked in a solution of 3% BSA in TBS (TBST without Tween-20). Blots were then washed twice in 0.02 M Tris, pH 7.5; 0.5 M NaCl; 0.5% Tween-20, 0.2% TritonX-100 (TBST-T) and washed once for 5 min in TBS. The blots were then incubated with the anti-peptide antibody at a dilution of 1:1000 in 3% BSA, TBS or with the anti-protein antibody at a dilution of 1:500 in 3% BSA, TBS for 1 hr. The blot was then washed one time quickly, one time for 10 min, and twice for 5 min each in TBST-T. The blots were then incubated with a donkey anti-rabbit IgG HRP – linked Whole Ab (Amersham Biosciences) at a dilution of 1:2000 in 5% milk, TBST for 1 hr. The blots were then washed in TBST-T one time quickly, one time for 10 min, and twice for 5 min. Two subsequent washes with TBST for 5 min were performed before

detection with ECL Western Blotting Detection Reagents (Amersham Biosciences) and exposure to X-ray film.

Protein Pulse Label and Pulse Chase Assays

Protein pulse labeling of infected cells with trans-^{35}S labeled methionine (ICN Biochemical), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and audoradiography were done as previously described (Condit et al., 1981). In protein pulse chase experiments the pulse-labeled cells were washed and incubated in serum containing medium for various times. At times 0, 3, 6, 9,12 hrs the cells were harvested and analyzed as described above.

Transcription by Permeabilized Virions

Permeabilized virion transcription experiments were done as previously described by Gershowitz and Moss (Gershowitz et al., 1979) and Gross and Shuman (Gross et al., 1996). To measure total incorporation into RNA reactions containing 60 mM Tris-HCl pH 8.0, 0.05% nonidet P-40 (NP40), 10 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 5 mM ATP, 1 mM UTP, 1 mM GTP, 0.2 mM α ^{32}P-CTP (3000 Ci/mmol Perkin Elmer) and 0.3 OD_{260} of virus were incubated at 30^{o}C, and 50 µl aliquots were precipitated directly with 5% TCA. The virion associated versus released RNA was measured using identical reactions except the 50 ul aliquots were diluted into 200 ul stop solution (50 mM Tris-HCl pH 8.0, 10 mM DTT, 10 mM EDTA, 0.05% NP40) and centrifuged for 3 min in a microfuge. The supernatants (released RNA) were then removed and precipitated in 5% TCA. The pellets (core associated RNA) were resuspended in 0.2mL of a buffer containing 50 mM Tris-HCl, pH 8.0, 0.1% SDS, and precipitated with 5% TCA. TCA perceptible radioactivity was determined by liquid scintillation counting.

CHAPTER 3
RESULTS

Mapping of the Mutations

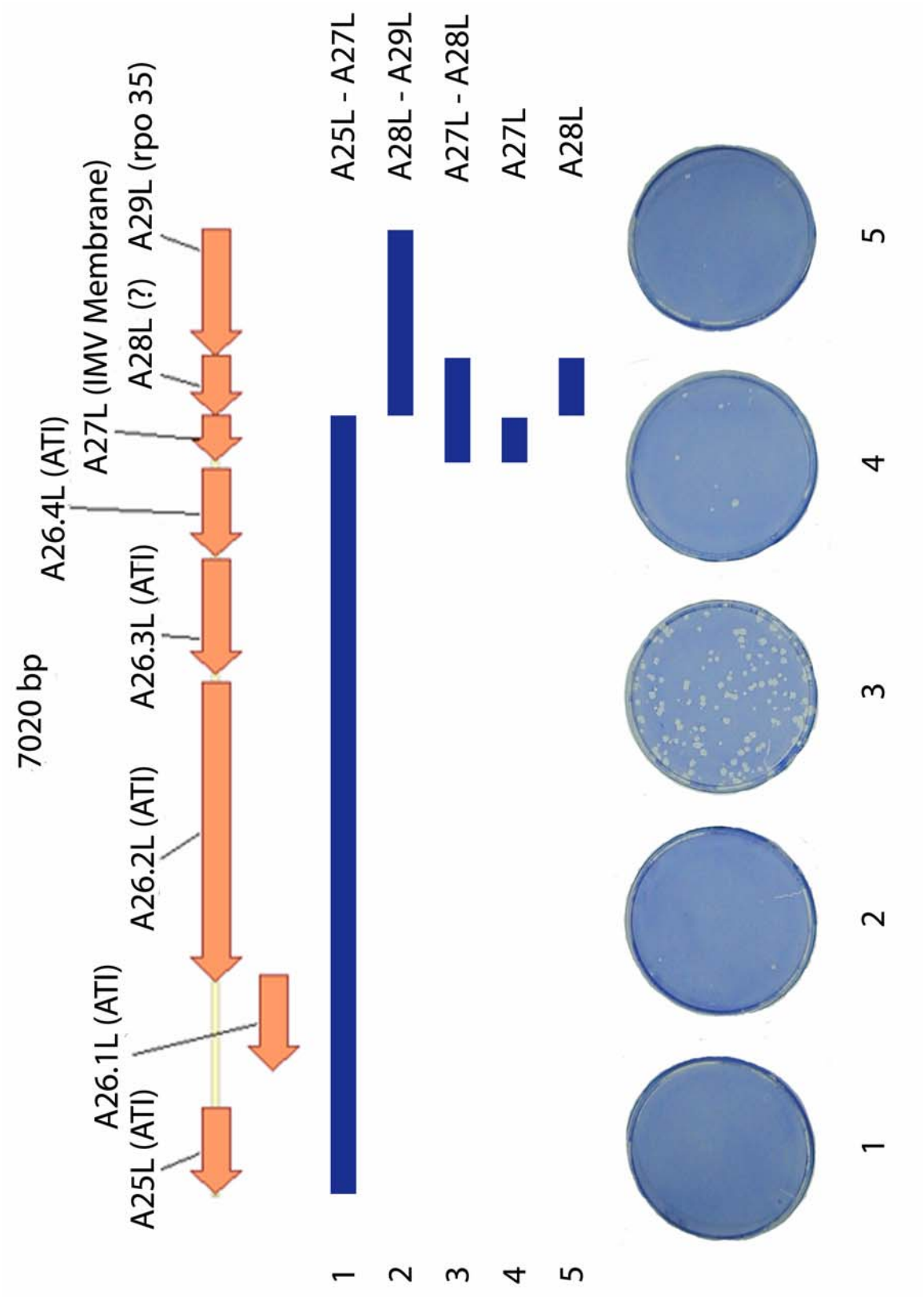
The temperature sensitive vaccinia virus mutants Cts6 and Cts9 comprise a complementation group that has a "normal" phenotype, that is, they display wild type protein and DNA synthesis (Condit et al., 1983). Not only were these mutants shown to be in the same gene by complementation analysis, but they were also shown to have an undetectable recombination frequency, $<1.6 \times 10^{-4}$, which suggests that the mutations lie close together (Condit et al., 1981). Using a library of overlapping cosmid clones, Thompson and Condit showed that Cts6 mapped to the right half of the HindIII A fragment of the vaccinia genome (Thompson et al., 1986). Further preliminary mapping (R. Condit, personal communication) showed that the mutation could be narrowed down to a 5 kB region of the genome. This approach used PCR products generated off vaccinia wild type DNA using primers designed and generously donated by B. Luttge and R Moyer. The genes included in this 5 kB region are the A25L – A26.4L (A-type inclusion proteins), A27L (IMV membrane protein), A28L (unknown function), and A29L (35 kD subunit of RNA polymerase) (Fig. 3). Unlike cowpox that encodes a functional A-type inclusion (ATI) gene, vaccinia virus has a homolog that is disrupted into several ORFs and is not functional. Because the A-type inclusion is likely to be non-essential and because of the preliminary phenotype of the mutants, A27L and A28L were the most likely candidate genes for the location of the mutation.

The first strategy that was used to rescue the mutants to a single ORF was to generate PCR products that specifically amplified each ORF, treating the ATI reading frames as a single ORF. These PCR products were then used in a one-step marker rescue experiment as described in the materials and methods. Attempts using this approach did not result in a significant rescue with any of the PCR products (data not shown). The next strategy taken was to generate overlapping PCR products that incorporated two or more genes. PCR products were made that specifically amplified A25L-A26.4L, A25L-A27L, A27L-A28L, A27L-A29L, and A28L-A29L ORFs. These PCR products along with the single ORF products were used in a one-step marker rescue experiment and several of the products resulted in significant rescue. Fig. 3 shows a subset of the results showing that rescue did not occur with products containing A25L-A27L, A28L-A29L, or with just A27L or A28L, however there is rescue with products that contains A27L and A28L. The experiment was performed with both Cts6 and Cts9 with similar results, however, only Cts6 is shown in Fig. 3. Because of the orientation of A27L and A28L this result suggests that the mutations must be at the 5' end of A27L or at the 3' end of A28L.

Sequencing Data

To determine the precise mutation that causes the temperature sensitivity in Cts6 and Cts9, the A27L and A28L genes from both viruses were sequenced. Figure 4 shows the nucleotide sequence alignment of A28L from Cts6, Cts9, and wt. The mutant viruses have wild type A27L genes and they both have a two nucleotide deletion in the A28L gene. Both mutants are missing the 394^{th} and 395^{th} nucleotides of the 438-nucleotide A28L gene, which in the wild type are both cytosine residues. This data is consistent with the marker rescue results, which suggested that the mutations could be located at the 3' end of A28L. The protein translation of Cts6 and Cts9 is shown in Fig. 5, which shows

Figure 3. Marker rescue of Cts6. At the top is a 7020 bp region of the vaccinia genome where Cts6 has been previously mapped, which includes genes A25L to A29L. The gene name and known protein function are indicated above each gene. The middle of the figure shows a schematic representation of a subset of PCR products used in the rescue of Cts6. The gene(s) included in each PCR product are indicated to the right of the product and each product is numbered to the left. The bottom of the figure shows a subset of dishes used in the rescue with the corresponding PCR product labeled below the dish.


7020 bp
A25L (ATI)
A26.1L (ATI)
A26.2L (ATI)
A26.3L (ATI)
A26.4L (ATI)
A27L (IMV Membrane)
A28L (?)
A29L (rpo 35)
A25L - A27L
A28L - A29L
A27L - A28L
A27L
A28L
1
2
3
4
5

```
                1                                                    50
Cts6-A28L     (1) ATGAACTCTCTATCAATTTTTTTTATTGTGGTAGCGACGGCTGCGGTGTG
Cts9-A28L     (1) ATGAACTCTCTATCAATTTTTTTTATTGTGGTAGCGACGGCTGCGGTGTG
  wt-A28L     (1) ATGAACTCTCTATCAATTTTTTTTATTGTGGTAGCGACGGCTGCGGTGTG
                51                                                   100
Cts6-A28L    (51) TTTACTTTTTATCCAGGGTTACTCAATATATGAAAATTATGGCAATATTA
Cts9-A28L    (51) TTTACTTTTTATCCAGGGTTACTCAATATATGAAAATTATGGCAATATTA
  wt-A28L    (51) TTTACTTTTTATCCAGGGTTACTCAATATATGAAAATTATGGCAATATTA
               101                                                   150
Cts6-A28L   (101) AGGAATTTAATGCTACTCATGCAGCATTCGAATATTCAAAATCTATAGGT
Cts9-A28L   (101) AGGAATTTAATGCTACTCATGCAGCATTCGAATATTCAAAATCTATAGGT
  wt-A28L   (101) AGGAATTTAATGCTACTCATGCAGCATTCGAATATTCAAAATCTATAGGT
               151                                                   200
Cts6-A28L   (151) GGAACACCGGCATTAGATAGGAGAGTTCAAGATGTCAACGACACAATTTC
Cts9-A28L   (151) GGAACACCGGCATTAGATAGGAGAGTTCAAGATGTCAACGACACAATTTC
  wt-A28L   (151) GGAACACCGGCATTAGATAGGAGAGTTCAAGATGTCAACGACACAATTTC
               201                                                   250
Cts6-A28L   (201) TGATGTAAAGCAAAAGTGGAGATGTGTGGTTTATCCAGGAAACGGTTTTG
Cts9-A28L   (201) TGATGTAAAGCAAAAGTGGAGATGTGTGGTTTATCCAGGAAACGGTTTTG
  wt-A28L   (201) TGATGTAAAGCAAAAGTGGAGATGTGTGGTTTATCCAGGAAACGGTTTTG
               251                                                   300
Cts6-A28L   (251) TATCCGCTTCCATATTTGGATTTCAGGCAGAAGTTGGACCCAATAATACT
Cts9-A28L   (251) TATCCGCTTCCATATTTGGATTTCAGGCAGAAGTTGGACCCAATAATACT
  wt-A28L   (251) TATCCGCTTCCATATTTGGATTTCAGGCAGAAGTTGGACCCAATAATACT
               301                                                   350
Cts6-A28L   (301) AGATCCATTAGAAAATTTAACACGATGCAACAATGTATAGACTTTACATT
Cts9-A28L   (301) AGATCCATTAGAAAATTTAACACGATGCAACAATGTATAGACTTTACATT
  wt-A28L   (301) AGATCCATTAGAAAATTTAACACGATGCAACAATGTATAGACTTTACATT
               351                                                   400
Cts6-A28L   (351) TTCTGATGTTATTAACATCAATATTTATAATCCATGTGTTGTA--AAATA
Cts9-A28L   (351) TTCTGATGTTATTAACATCAATATTTATAATCCATGTGTTGTA--AAATA
  wt-A28L   (351) TTCTGATGTTATTAACATCAATATTTATAATCCATGTGTTGTACCAAATA
               401                                  438     ^^
Cts6-A28L   (399) TAAATAACGCAGAGTGTCAGTTTCTAAAATCTGTACTT
Cts9-A28L   (399) TAAATAACGCAGAGTGTCAGTTTCTAAAATCTGTACTT
  wt-A28L   (401) TAAATAACGCAGAGTGTCAGTTTCTAAAATCTGTACTT
```

Figure 4. Nucleotide alignment of A28L from wild type, Cts6, and Cts9 viruses. In Cts6 and Cts9 cytosine residues located a positions 394 and 395 are deleted, which are indicated by a ^ below the sequence.

A

```
                1                                                    50
Cts6-A28   (1)  MNSLSIFFIVVATAAVCLLFIQGYSIYENYGNIKEFNATHAAFEYSKSIG
Cts9-A28   (1)  MNSLSIFFIVVATAAVCLLFIQGYSIYENYGNIKEFNATHAAFEYSKSIG
  wt-A28   (1)  MNSLSIFFIVVATAAVCLLFIQGYSIYENYGNIKEFNATHAAFEYSKSIG
                51                                                   100
Cts6-A28  (51)  GTPALDRRVQDVNDTISDVKQKWRCVVYPGNGFVSASIFGFQAEVGPNNT
Cts9-A28  (51)  GTPALDRRVQDVNDTISDVKQKWRCVVYPGNGFVSASIFGFQAEVGPNNT
  wt-A28  (51)  GTPALDRRVQDVNDTISDVKQKWRCVVYPGNGFVSASIFGFQAEVGPNNT
                101                                                  146
Cts6-A28 (101)  RSIRKFNTMQQCIDFTFSDVININIYNPCVVKYK-----------
Cts9-A28 (101)  RSIRKFNTMQQCIDFTFSDVININIYNPCVVKYK-----------
  wt-A28 (101)  RSIRKFNTMQQCIDFTFSDVININIYNPCVVPNINNAECQFLKSVL

                     ^
```

B

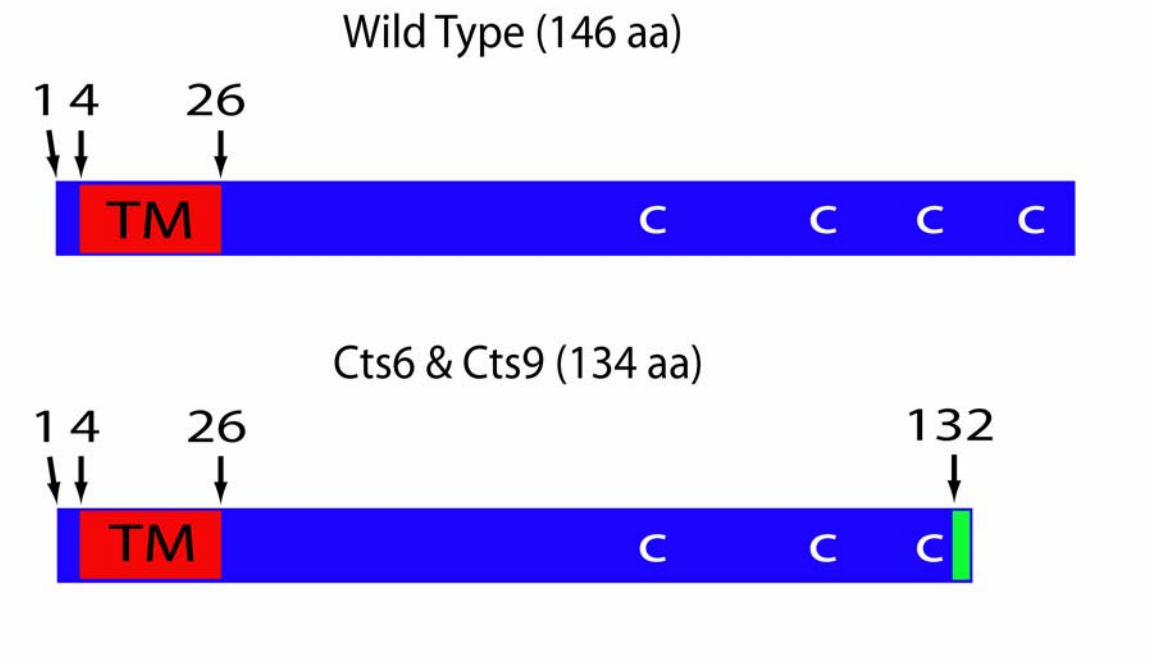


Figure 5. Amino acid sequence alignment of A28 protein from wild type, Cts6, and Cts9 (A). The first 131 amino acids encoded are identical in mutant and wild type viruses. The mutation, which is indicated by a ^ under the sequence, causes a frameshift which results in three aa substitutions followed by a stop codon, which causes a C-terminal truncation. (B) Wild type and mutant A28 proteins. A transmembrane domain, indicated by a red box and labeled TM, is located between the 4th and 26th amino acids. The green box at the C-terminal end of the mutant protein represents the three substitutions encoded by the mutant viruses, which begins at aa 132. The Cs inside the proteins represent conserved cysteines (see discussion).

that the mutants produce C-terminal truncated proteins. The wild type protein is 146 amino acids (aa) in length, while the mutant form of the protein is only 134 aa in length. The two deletions cause a frameshift and after the first 131 aa are translated correctly 3 aa are substituted before a stop codon is reached.

Protein Synthesis

Cts6 and Cts9 have been previously shown to have a "normal" phenotype due to their wild type levels of DNA and protein synthesis (Condit et al., 1983). In order to confirm the protein synthesis results both mutants were assayed for their pattern of protein expression in infected cells. Cells were infected with Cts6, Cts9, or wt and incubated at 31^{o}C or 40^{o}C, and proteins were pulse-labeled at various times post-infection with ^{35}S-methionine. The proteins were then separated by SDS-PAGE and detected by autoradiography (Fig. 6). The wt virus represents normal protein synthesis at 31^{o}C or 40^{o}C. Both Cts6 and Cts9 show protein synthesis that looks like wild type. Cell host proteins are seen at 0 h post-infection and are subsequently shut off by 3 h post-infection with the appearance of early proteins. Late viral protein synthesis is seen by 6 h post-infection and persists throughout the experiment. The mutants displayed a normal pattern of protein synthesis, however, some of the films appear lighter due to a technical problem during exposure.

Protein Processing

The processing of several viral proteins is associated with morphogenesis from IVN to IMV (Klemperer et al., 1997). Virus morphogenesis is arrested at the IVN stage with a mutation in a protease that is implicated in cleavage of virion proteins (Byrd et al., 2002;Kane et al., 1993). Therefore, virion protein processing is a good marker for detecting defects in virus assembly. In order to test whether the major core polypeptides

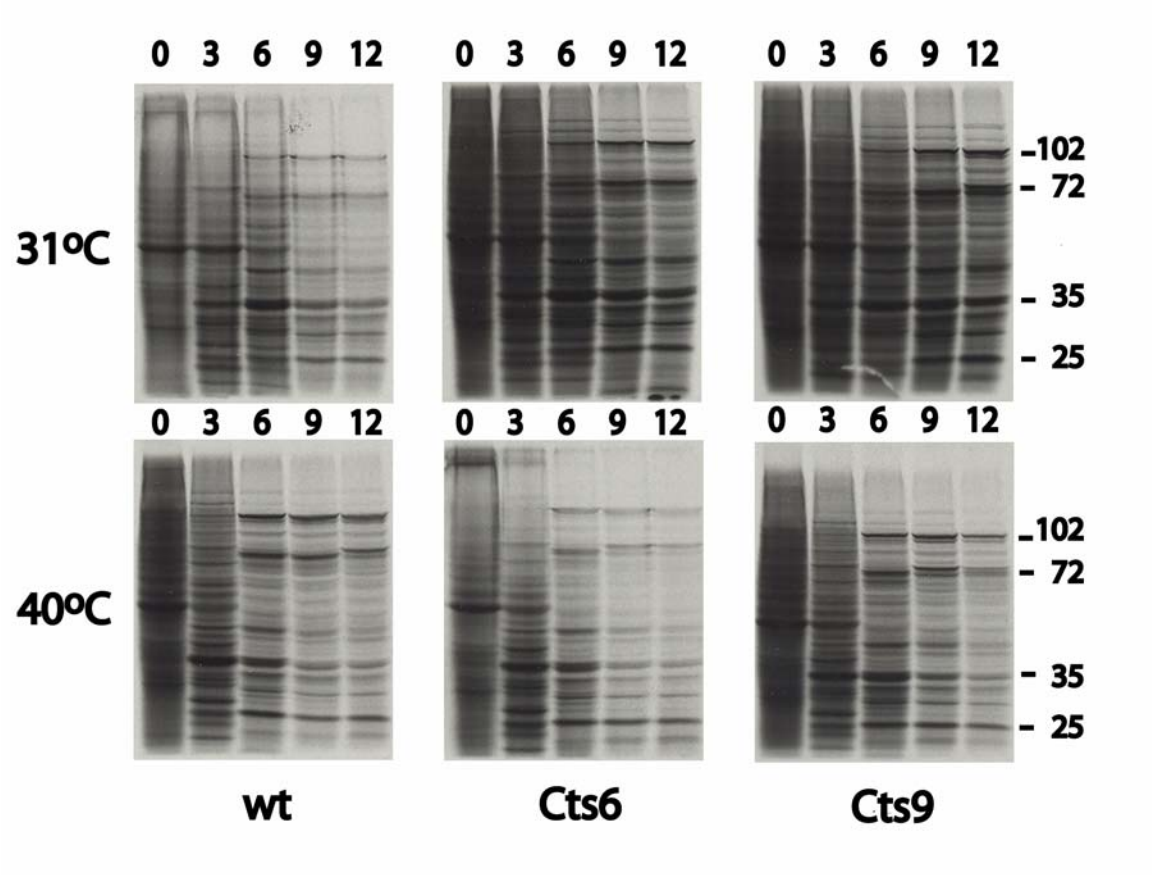


Figure 6. Viral protein synthesis in wild type, Cts6, and Cts9 infected cells. Cells were infected at 31°C and 40°C, and pulse-labeled for 15 min with trans-^{35}S methionine. Cells were harvested, and the proteins were separated by a 10% SDS-PAGE and autoradiographed. The autoradiograms are shown above. The infecting virus is indicated along the bottom, and temperature of incubation is indicated at the left. Approximate molecular weights, in kilodaltons, are shown to the right of the gels. The time of the pulse labeling is indicated, in hours, above each lane.

are processed correctly Cts6 and Cts9 were assayed in a pulse chase experiment. Cells were infected with wt, Cts6, or Cts9 and incubated at 31^{o}C or 40^{o}C for 8 h and pulse labeled with ^{35}S-methionine. At various times after labeling the cells were chased in the presence of unlabeled amino acids, harvested, and analyzed by SDS-PAGE and autoradiography (Fig. 7). Normal proteolytic processing can be observed by the decrease in intensity of the p4a and p4b proteins, 102 and 72 kD respectively. The products of these cleavages result in the appearance of two more bands 4a and 4b, 62 and 60 kD respectively. The results show that Cts6 and Cts9 are able to process p4a and p4b into their respective products 4a and 4b. The disappearance of the 102 kD band is not as prominent as usual. This could be a result of an insignificant removal of the label, which was gently done only once to ensure the integrity of the infected cell monolayer. However the appearance of the 60 and 62 kD bands are strong evidence that the proteins are being processed normally.

Electron Microscopy

Transmission electron microscopy was used to further investigate Cts6 and Cts9 to see if they are able to assemble normal looking particles *in vivo*. Briefly, BSC40 cells were infected with wt, Cts6, or Cts9 at 31^{o}C or 40^{o}C for 24, 36, or 48 h and then fixed and processed for electron microscopy. Figures 8 & 9 show that Cts6 and Cts9 are indistinguishable from wt at both 31^{o}C and 40^{o}C. All stages of vaccinia virus morphogenesis were observed including viral crescents, immature virions, immature virions with nucleoids, and intracellular mature virions.

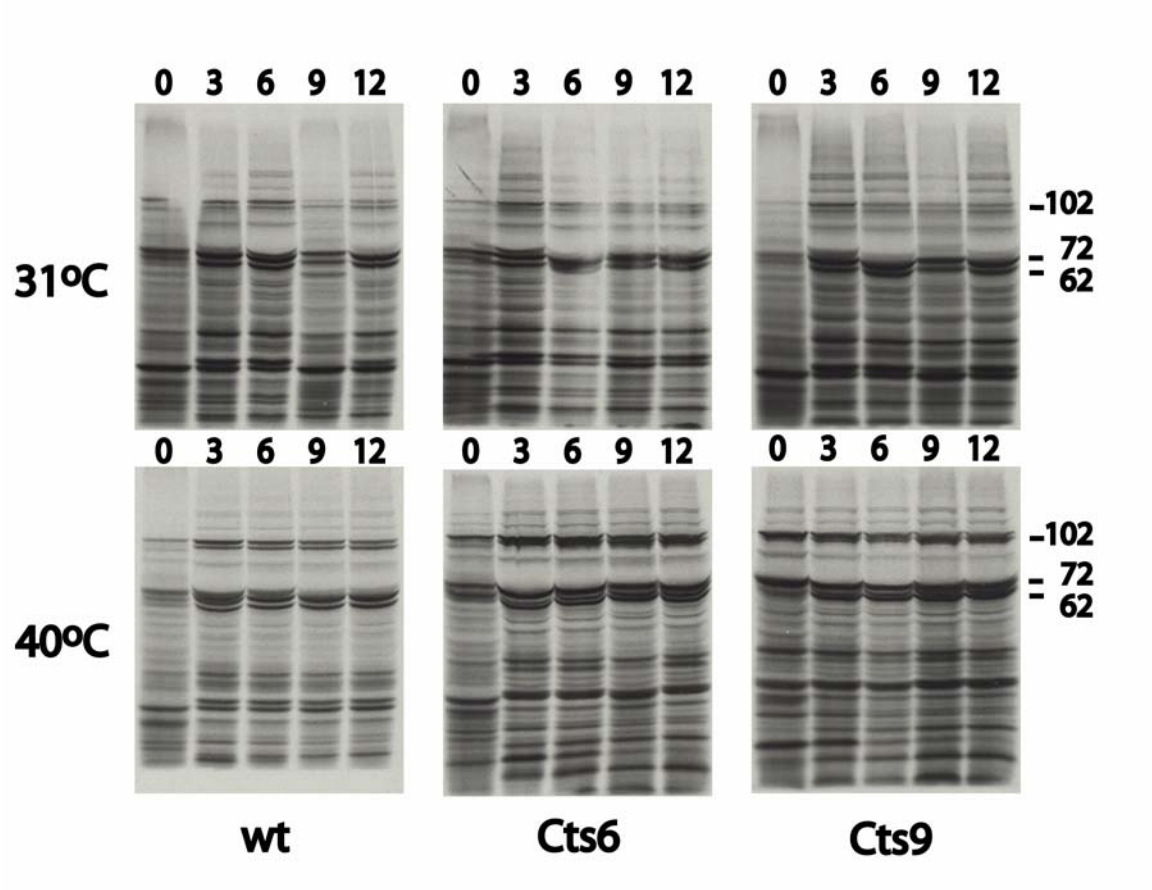
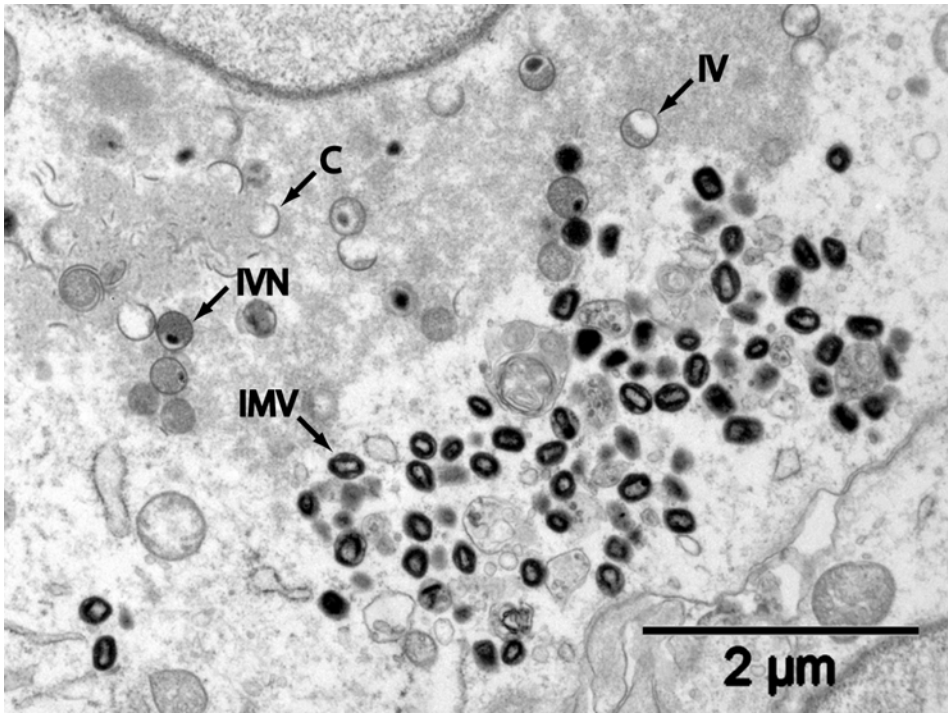


Figure 7. Viral protein processing in infected cells. 8 hours after infection cells were labeled with trans-^{35}S methionine, then chased with unlabeled amino acids for up to 12 hours. Cells were harvested and separated on a 12% SDS-PAGE and autoradiographed. The autoradiograms are shown. The infecting virus is indicated along the bottom, and temperature of incubation is indicated at the left. Approximate molecular weights, in kilodaltons, are shown to the right of the gels. The length of the chase is indicated, in hours, above each lane.

A



B

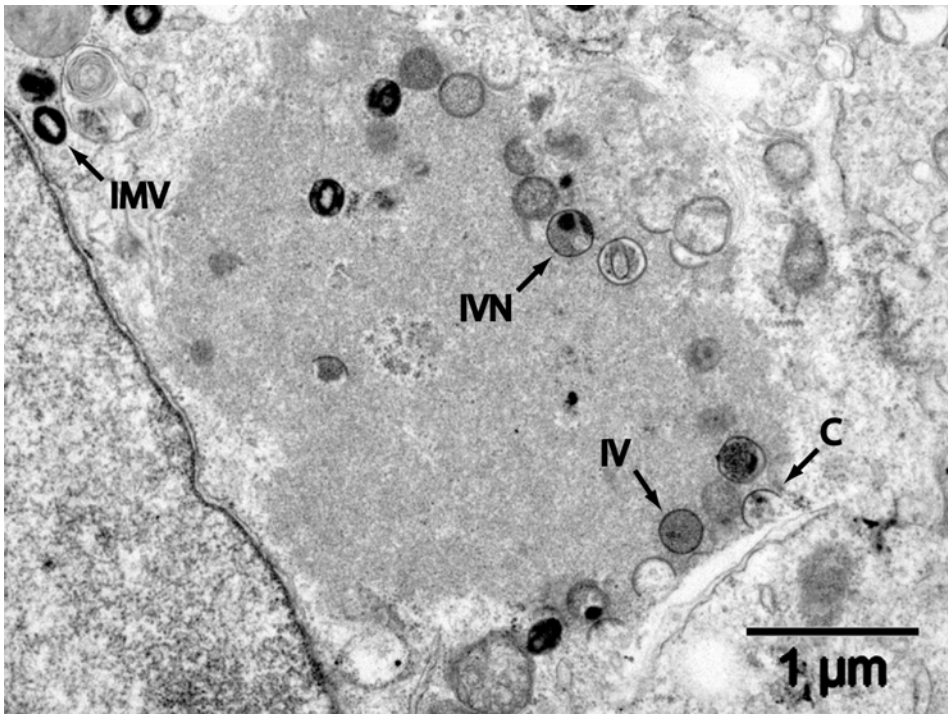
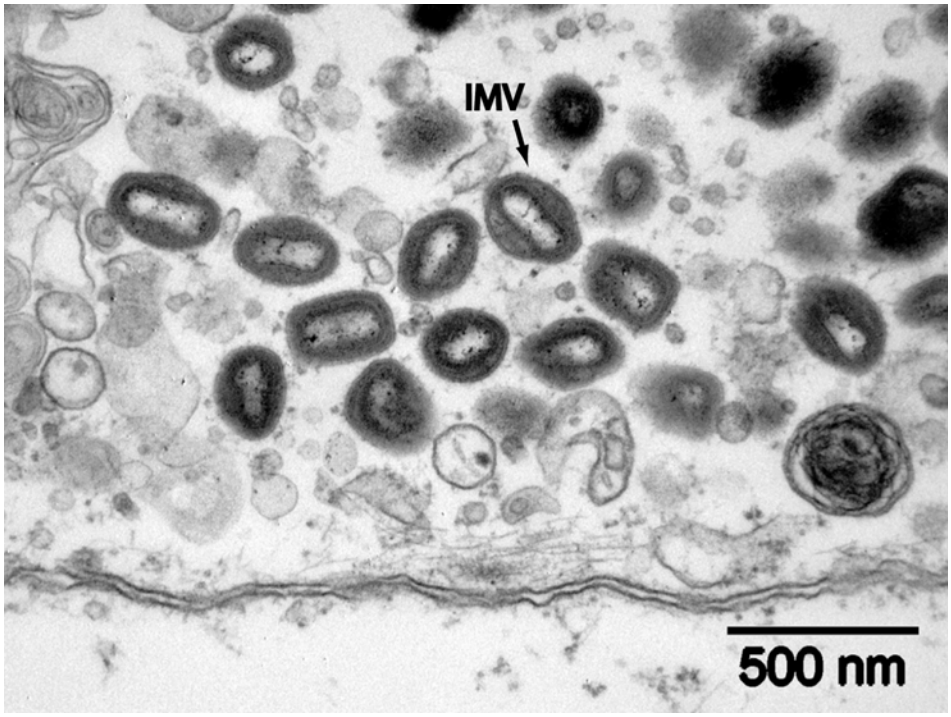


Figure 8. Electron micrograph pictures of cells infected with Cts6 (A) or wt (B) at 40 ^{o}C. Cells were infected with an m.o.i. of 10 and processed for electron microscopy 24 h postinfection. Arrows indicate examples of the different stages of morphogenesis: cresents (C), immature virions (IV), immature virions with nucleoid (IVN), and intracellular mature virion (IMV).

A



B

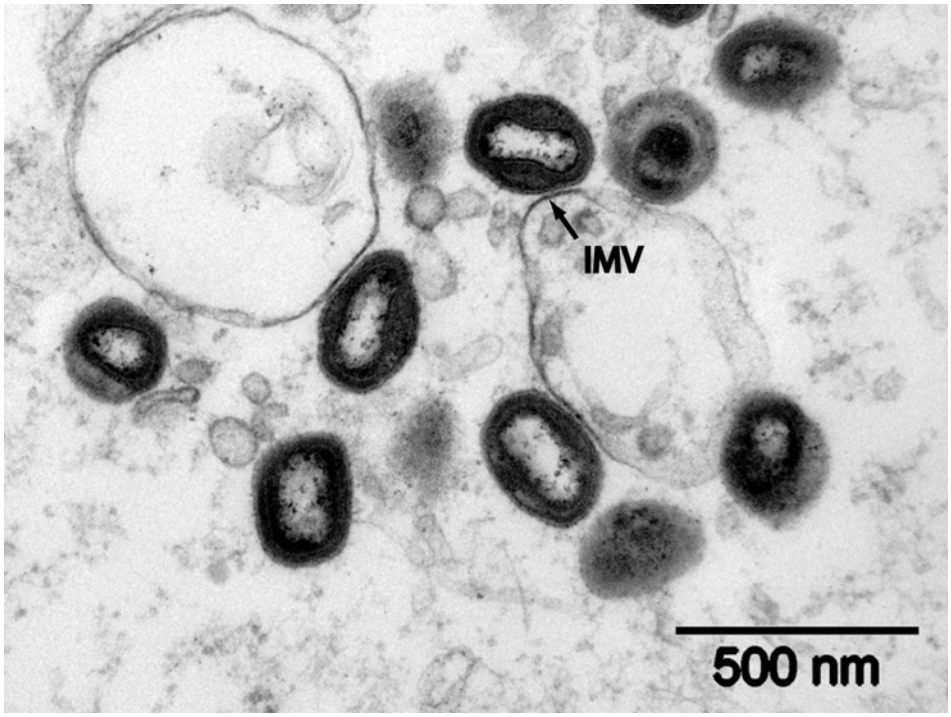


Figure 9. Electron micrograph pictures of cells infected with Cts9 (A) or wild type (B) at 40 °C. Cells were infected with an m.o.i. of 10 and processed for electron microscopy at 24 h postinfection. The arrow points to an example of an intracellular mature virion (IMV).

Immunodetection of A28 Protein

To further characterize the mutants, they were examined by western blot using A28 specific antibodies. Two antibodies were used in these western blots, an anti-peptide antibody and an anti-protein antibody, prepared as described in materials and methods. Infected cell lysates were first examined with anti-protein antibody. Briefly, cells were infected with wt, Cts6, or Cts9 and incubated at 40^{o}C for various times, then harvested and the proteins separated by SDS-PAGE. After transferring the proteins to a nitrocellulose membrane the blots were incubated with a 1:500 dilution of anti-protein antibody, then incubated with an anti-rabbit antibody and detected with ECL detection reagent and autoradiography (Fig. 10 A).

A band the correct size for A28, about 15 kD, was detected in all three viruses late in the infection. This band was not seen in the mock, 3 h post infection, or 6 h post infection lanes, however it was seen in the 9 h post infection and the 12 h post infection lanes. The viruses were grown at 40^{o}C and purified, as described in materials and methods and were examined with the anti-peptide antibody. Briefly, 0.2 OD units of purified virus was run on a 10-20% gradient gel, and transferred to a nitrocellulose membrane. The membrane was incubated with a 1:1000 dilution of the anti-peptide antibody, then incubated with a secondary anti-rabbit antibody and finally detected with ECL detection reagent and autoradiography (Fig. 10 B). A band the correct size was detected in purified virions in all three viruses. The two mutant viruses seemed to contain a slightly smaller protein, which would be consistent with the sequencing data. The amount of A28 protein in the mutant viruses also seemed to be much less than wild type.

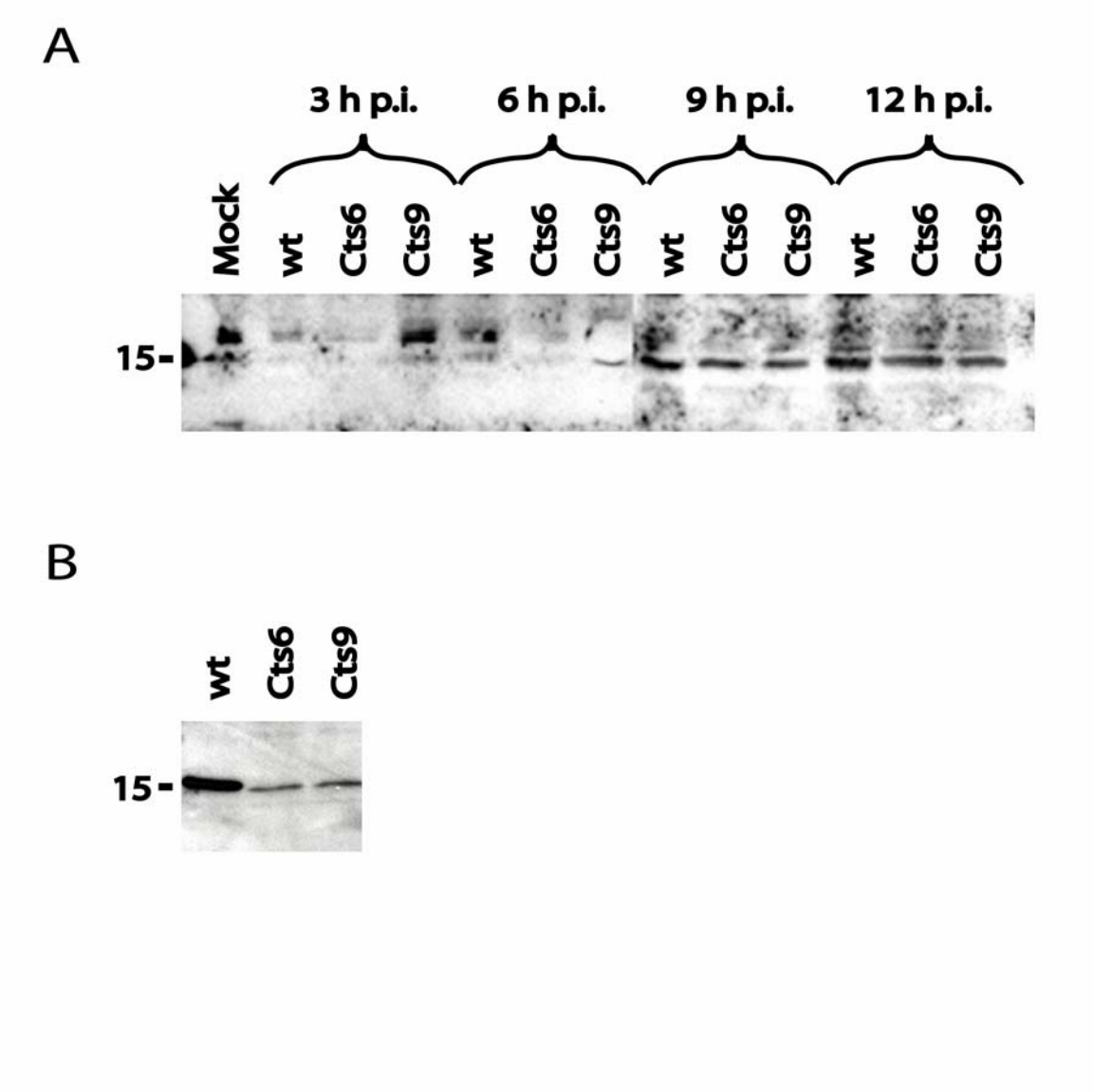


Figure 10. Immunodection of A28 protein. (A) Western blot of infected cell time course. Proteins were separated on 12% SDS-PAGE and detected with anti-protein antibody. The infecting virus and time harvested after infection, in hours, is indicated above gel. Approximate molecular weight marker, in kilodaltons, is shown on the left. (B) Western blot of purified virus. Proteins were separated on a 10-20% gradient gel and detected with anti-peptide antibody. The purified virus is indicated above the gel and approximate molecular weight, in kilodaltons, is shown on the left.

Virion Transcription

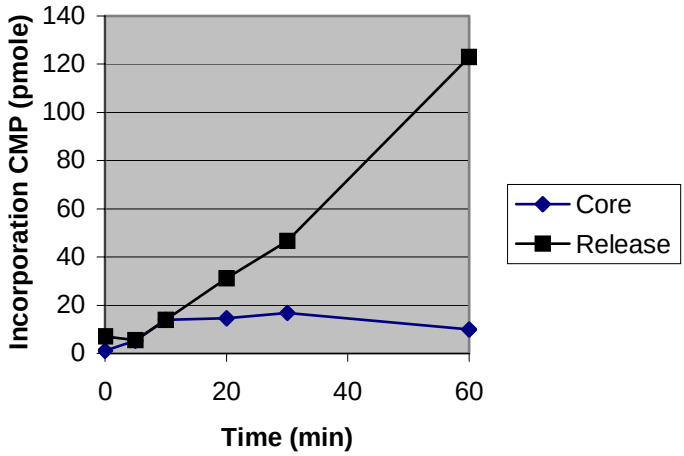
Purified vaccinia particles can be treated with NP40 and DTT to permeabilize them by removing the outer membrane, but leaving the enzyme-containing core structurally and functionally intact. These permeablilized virions are capable of carrying out transcription and modification of early viral mRNA (Hassett et al., 1997). Purified virions that were grown at 40^{o}C, as described in materials and methods were permeabilized with a NP40 and DTT solution and incubated with NTPs, and α ^{32}P-CTP at 30^{o}C for various times. At each time point the cores were spun down and the supernatant, which contained released transcripts, was TCA precipitated, filtered, and counted in a liquid scintillation counter. The cores were then disrupted in a SDS solution, TCA precipitated, filtered, and counted in a liquid scintillation counter. The results (Fig. 11) show that the mutants are able to synthesis and release early mRNA. The mutants do show a reduced amount of mRNA compared to the wild type, however it is only about a 2-fold difference.

Particle to Infectivity Ratio

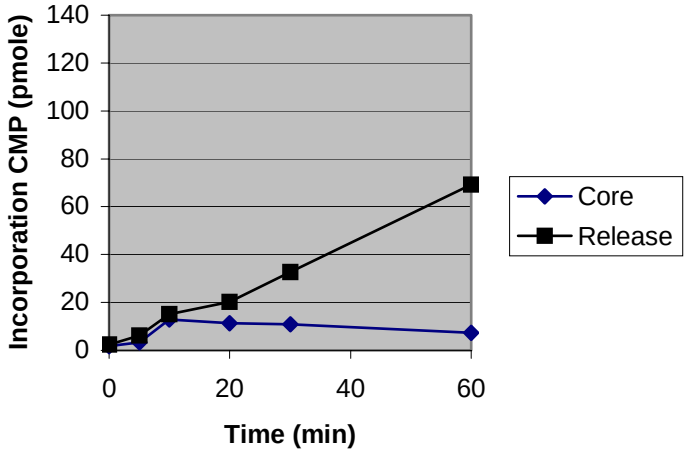
In order to further study Cts6 and Cts9, purified particles grown at 40^{o}C were assayed at 31^{o}C to see if they were infectious at the permissive temperature. Briefly, BSC40 cells were infected with a serial dilution of purified virus, and grown at 40^{o}C for seven days in order to determine plaque forming units per mL (pfu/mL). Using the standard 1 OD_{260} = 1.2 x 10^{10} particles/mL (Joklik, 1962a), the concentration of virus particles was determined. The particle to infectivity ratio was then calculated for wt, Cts6, andCts9. Wt virus has a particle to infectivity ratio of 39.2 particles/plaque forming unit. Cts6 has a ratio of 11,200 particles/plaque forming unit. Cts9 has a ratio of 10,000 particles/plaque forming unit. This suggests that mutant viruses are almost 250 fold less infectious as the wild type virus.

Figure 11. *In vitro* transcription activity of wild type (A), Cts6 (B), and Cts9 (C) purified virions. Purified vaccinia virus particles were incubated for various times, indicated on the x axis, with detergent and ribonucleside triphospahtes The incorporation of α ^{32}P-CTP into RNA was determined, which is indicated on the y axis.

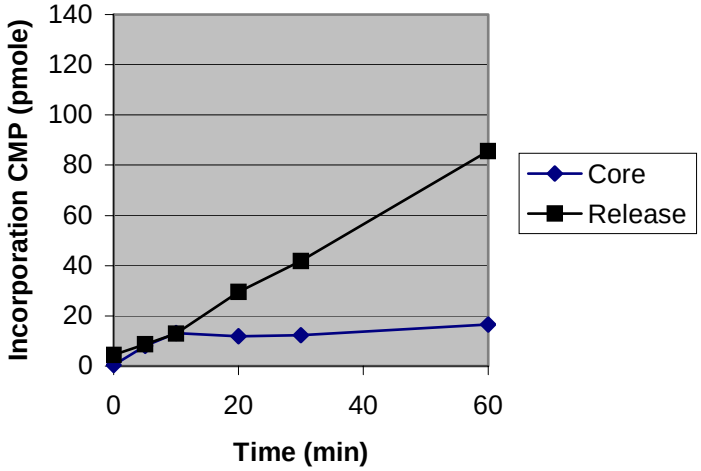
A


Incorporation CMP (pmole)
140
120
100
80
60
40
20
0
0
20
40
60
Time (min)
Core
Release

B


Incorporation CMP (pmole)
140
120
100
80
60
40
20
0
0
20
40
60
Time (min)
Core
Release

C


Incorporation CMP (pmole)
140
120
100
80
60
40
20
0
0
20
40
60
Time (min)
Core
Release

CHAPTER 4
DISCUSSION

Temperature sensitive (ts) vaccinia mutants have played a crucial role in the understanding of vaccinia virus morphogenesis. Ts mutants have implicated many genes involved in viral morphogenesis including H5 (Demasi et al., 2000), F10 (Wang et al., 1995), J1 (Chiu et al., 2002), and I6 (Grubisha et al., 2003). The purpose of this study was to use the ts vaccinia virus mutants Cts6 and Cts9 in order to further our understanding of morphogenesis. Cts6 and Cts9 are a result of hydroxylamine mutagenesis and random plaque screening. They have been previously described to have a "normal" phenotype, based on their ability to synthesize DNA and proteins at wild type levels (Condit et al., 1983), which makes them candidates for defects in viral morphogenesis.

The genetic mapping of the mutants to a single open reading frame was the first goal of this project. Cts6 and Cts9 have been previously mapped by marker rescue to the right side of the HindIII A fragment (Thompson et al., 1986). R. Condit further mapped Cts6 and Cts9, using PCR products generated with primers donated by R. Moyer and B. Luttge, to a 5 kB region of the vaccinia genome shown at the top of Fig. 3. This region of the genome includes genes A25L through A29L. Some poxviruses, such as cowpox and fowlpox, encode a protein that causes IMVs to become occluded in a dense protein matrix called A-type inclusions (ATI). This ATI protein is not essential and vaccinia has a frameshift mutation that disrupts the ATI gene into five smaller ORFs (Cooper et al., 1981). These ORFs are named A25L, A26.1L, A26.2L, A26.3L, and A26.4L and were

treated as a single ORF for mapping purposes. These genes were not prime candidates for containing the mutation, because ATI is not an essential protein. The A29L gene encodes the 35 kD subunit of the RNA polymerase. A29L was also thought not to contain the mutation because Cts6 and Cts9 were shown to display normal protein synthesis (Condit et al., 1983), while an RNA polymerase mutant is defective in the synthesis of late viral mRNA and proteins (Hooda-Dhingra et al., 1989). The two candidate genes left in the region are A27L and A28L, which are the two genes most likely to contain the mutations. A27L protein encodes a IMV protein involved in morphogenesis (Sanderson et al., 2000). Nothing was known about the A28L gene at the time of the mapping. PCR products that specifically amplified each open reading frame in this 5 kB region were used in a marker rescue experiment but was unsuccessful. The next approach used was to create PCR products that contained more than one ORF. PCR products were made that included A25L-A27L, A27L-A28L, A27L-A29L, and A28L-A29L, and used in another maker rescue along with the single ORF products. The mutants could only be rescued with products that span both A27L and A28L, but not with either gene individually. Both A27L and A28L are oriented to the left, suggesting that the mutations lie either at the beginning of A27L or at the end of A28L.

The region spanning A27L and A28L from wt, Cts6, and Cts9 was sequenced and aligned. TheA27L gene was identical in all three viruses. Differences were found between the wt and mutants sequences in the A28L gene. Surprisingly the mutant viruses have the exact same sequence for the A28L gene. Several explanations exist for both mutants having identical A28 sequences; the most likely of them is that these viruses are siblings generated by spontaneous mutations and were present in the wild type virus

stock used in the mutagenesis. The mutations comprise deletion of the 394th and 395th nucleotides of the A28L gene. A28L is present in all poxviruses and with its high sequence conservation it is predicted to play an essential role in the virus life cycle (Senkevich et al., 2004a). This result fits our marker rescue results well because the first deletion is only 45 nucleotides away from the stop codon. This data is the best example from our lab of the amount of overhang required for a successful recombination in vaccinia virus. The translation of the mutant A28 protein results in a C-terminally truncated protein. The mutations cause a frameshift after the first 131 aa are translated there are three aa substitutions before a stop codon is reached.

As discussed earlier, Cts6 and Cts9 have a preliminary phenotype of "normal," which means they are able to synthesize DNA and proteins at wild type levels. Before further characterization of the mutants could take place confirmation of these preliminary results was completed. In order to look at the protein synthesis of the mutants a pulse label assay, as described in materials and methods, was performed on the mutants. Cts6 and Cts9 display a normal pattern of protein synthesis, confirming the preliminary protein synthesis results. Because late protein synthesis requires DNA replication (Moss, 2001) these results are sufficient to confirm the normal DNA synthesis phenotype.

Viral protein processing of core proteins such as 4a, 4b, and p25 coincides with the maturation of the IVN into IMV (Sodeik et al., 2002). The next step in the characterization of the mutants was to check the mutants for their ability to process these core proteins. Although we were anticipating the mutants to be defective, they displayed wild type levels of protein processing. These results suggest that any morphogenesis defect present in Cst6 and Cts9 exists after the formation of IMVs.

Because the mutants are able to process viral core proteins we wanted to examine the viral particles under an electron microscope to determine whether normal looking IMVs were made. We also wanted to see if we could discover any other defects in the morphogenesis pathway the mutants might contain. The mutants appeared normal throughout all the stages of viral morphogenesis at the nonpermissive temperature. Mutant IMVs made at the nonpermissive temperature were then purified for further study.

Purified particles and infected cells were checked for the presence of the A28 protein. Cts6 and Cts9 have the same protein expression pattern of A28 as wild type virus in infected cells. The A28 protein is expressed at late times during a vaccinia infection. Sequence analysis of A28L reveals that the A of the start codon is part of a TAAA, which is a typical promoter of poxvirus late genes (Davison et al., 1989). A28 contains an early transcription termination sequence, TTTTTAT, prohibiting A28 expression at early times (Ink et al., 1989). Analysis of 0.1 OD units of purified virions revealed that the mutants are able to package the A28 protein into the virion, however, less protein is present than in wild type virions. Because the mutants cannot package A28 at wild type levels they may have a morphogenesis defect during assembly. The mutant protein that is packaged into the virion is slightly smaller than the wild type protein, which was predicted by the sequencing data. This suggests that the mutant protein is stable, however, it is not functional at the nonpermissive temperature.

All the enzymes necessary for transcription and modification of early mRNAs are packaged into the virion. Thus, it is possible to permeabilize purified particles with a neutral detergent and incubate them with nucleoside triphosphates and they will produce

authentic fully modified early mRNAs *in vitro* (Condit et al., 2002). Cts6 and Cts9 purified particles are both able to transcribe mRNAs *in vitro*, however, only about half as efficient as wild type. This is further evidence that although the mutant particles appear normal they may not be assembled correctly resulting in slightly diminished transcription.

The existence of multiple infectious forms of virus has complicated the study of virus entry. IMVs are the form of virus usually used by investigators to infect cells in the laboratory and it is possible to purify these particles from infected cells to study them (Moss, 2001). The purified IMV particles made at the nonpermissive temperature were assayed to see if they are able to infect cells at the permissive temperature. We found that the mutant particles were about 1,000 times less infectious than wild type particles. Cts6 and Cts9 are capable of all viral functions once inside of the cell. However, mutant virions made at the nonpermissive temperature are not infectious at the permissive temperature. This suggested to us that the mutants are not able to enter the cell, which means that A28 has a role in virus entry into the cell.

Very recently Senkevich et al. (2004) published two papers about A28L and its role in vaccinia biology. The papers used an A28 hemagglutinin tagged inducible knockout virus to demonstrate many of the same results we have shown. A28 is expressed as a late protein, is essential for virion infectivity, and replication. A28 is not required for RNA and protein synthesis, protein processing, or the assembly of intracellular and extracellular virions. Further investigation revealed A28L to be a substrate of the viral disulfide bond formation enzyme and other aspects that our study did not address. A28 was shown to be anchored in the IMV membrane by a N-terminal transmembrane domain, while the C-terminus is exposed on the surface and is predicted

to contain two intramolecular disulfide bonds at four conserved cysteines. An extensive investigation of A28's role in virus entry revealed that it is not required for binding to the cell, however, A28 is required for cell rounding and cytopathic effects. A28 deficient virions have a defect in penetration and membrane uncoating. The A28 deficient virions also were not able to cause vaccinia virus-induced cell fusion (Senkevich et al., 2004b;Senkevich et al., 2004a).

The predicted intramolecular bonds of A28 are relevant to our studies using Cts6 and Cts9. The conserved cyteines thought to be involved in the bonds are shown in Fig. 5. The terminal cysteine is not present in the mutant protein, which means that one of the disulfide bonds cannot be formed and may have a functional effect on the protein. Further investigations with Cts6 and Cts9 need to be conducted to see if any differences are present between the ts mutants and the A28 knockout virus. Vaccinia virus-induced cells fusion is thought to mimic events during virus entry, and A28 is required for virus-induced cell fusion (Senkevich et al., 2004b). This suggests that A28's role in virus entry is through fusion with the plasma membrane. A27 is another IMV protein that has been implicated in cell fusion (Vazquez et al., 1998). A27 and A28 may work together in fusion and entry, however, A27-deficient IMV retain nearly complete (Hsiao et al., 1999) or partial (Vazquez et al., 1999) infectivity. A27 has also been shown to function in binding to cell surface glycoaminoglycans (Vazquez et al., 1999). Although the mechanism of action is still undetermined, A28 is an essential vaccinia protein that functions in cell fusion and entry.

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BIOGRAPHICAL SKETCH

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