ROLE OF THE E6 GENE IN VACCINIA VIRUS MORPHOGENESIS

By

OLGA BOYD

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Olga Boyd

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The E6R gene of vaccinia virus is an essential structural gene as suggested by preliminary experiments; however the function of E6 is unknown. To investigate the role of the E6R gene during vaccinia virus infection, two mutants, the temperature sensitive mutant Cts52 and the inducible mutant vE6i, each defective in synthesis of normal E6 protein under non-permissive conditions, have been analyzed. The results show that growth of both mutants is inhibited under non-permissive conditions; however both mutants are normal for DNA replication and virus gene expression. Protein processing of the major precursor proteins required for formation of mature virions is normal in the Cts52 mutant and absent in the vE6i mutant. Electron microscopy of Cts52 showed production of mature virions under non-permissive conditions which are indistinguishable from wild type mature virions in appearance, but vE6i formed empty immature virions under non-permissive conditions, and failed to make mature virions suggesting block in morphogenesis. In addition, dense "crystalloid" viroplasm was observed in cytoplasm of vE6i infected cells; crystalloids probably represent deposits of unpackaged viral DNA. Analysis of infected cells for viral DNA concatemer resolution showed DNA resolution occurs normally. vE6i infection blocked in morphogenesis was analyzed for the ability to package the existing DNA and complete morphogenesis when permissive conditions were introduced. Thus,

permissive conditions with DNA replication inhibitor introduced at 24 hours post-infection to cells infected under non-permissive conditions and which therefore had presumably formed crystalloids; the induction of E6 expression resulted in increased virus yield, suggesting formation of mature virions via processing of existing viral DNA. Finally, purified Cts52 viral particles grown under non-permissive conditions are defective in core transcription in vitro. A dual role of E6 gene in infection suggested the following: 1) in the virion core E6 may have a subtle direct or indirect role in viral transcription; 2) E6 is essential for association of viroplasm and crescents to encapsidate DNA into immature virions. Understanding E6R gene function will advance our knowledge of vaccinia virus structure and assembly.

CHAPTER 1
INTRODUCTION

Vaccinia Virus Overview

The poxvirus family has had an enormous impact on world history and medicine. One member of the poxvirus family, the variola virus, caused smallpox, the disease that devastated humanity for centuries and wiped out millions of lives; those who survived were left scarred for life. To conquer this deadly disease, another member of the same family, vaccinia virus, was used as the live vaccine to eradicate smallpox entirely worldwide by 1977 and gave roots to the general practice of vaccination which has saved millions of lives. Nowadays, smallpox is extinct as disease, but important lessons can be learned by dissecting the virus structure and the function of each viral gene. The unique structure of poxvirus has attracted many scientists and led to extensive research in the past few decades and because poxviruses are conserved throughout the entire family, vaccinia has become the prototype virus used to study poxviruses.

The vaccinia virus belongs to the *Orthopoxvirus* genus of *Poxviriridae* family. Vaccinia is truly a unique virus with no parallel among other viruses in its shape, structure, replication and assembly of new viral particles. The brick shaped virus contains a large double-stranded (ds) DNA genome that encodes all of the factors for the virus' exclusively cytoplasmic replication. The assembly of new virus occurs in subsequent stages that involve assembly of viral membranes into spheres, encapsidation of the genome and morphogenesis into a mature viral particle. The ultimate goal of our study is to expand and deepen our knowledge of all aspects of vaccinia virus assembly.

Virus Structure

Vaccinia is unparalleled in its shape and structure compared to any other known viruses. Knowledge about the virus surface and the internal structure is gained from electron microscopy

(EM). Figure 1-1 shows electron micrographs of vaccinia virion that exemplifies virus surface and viral internal structure. In contrast to icosahedral and helical shaped viruses, vaccinia virus has a brick-shaped form (Figure 1-1A) with particle dimensions of 310 x 240 x 140 nm (Griffiths et al., 2001; Roos et al., 1996; Sodeik and Krijnse-Locker, 2002). The virus structure is complex and unusual among viruses. Cryo-section of the viral particle, shown on Figure 1-1B, reveals an outer lipid bilayer membrane that surrounds a dense biconcave core flanked by lateral bodies. Based on EM pictures, a virus model was constructed (Figure 1-2). The model is highlights the major elements of the virion structure as a brick shape, a core with lateral bodies and a genome arranged in tubules. The core contains a large double-stranded DNA genome approximately 200 kb in length that comprises 200 intronless genes, each gene controlled by its own promoter (Moss, 2007). The genome is organized into a linear molecule that contains inverted terminal repeats (ITR) with hairpin loops on both ends that are essential in viral DNA replication. The virion core also contains numerous viral enzymes including a multi-subunit RNA polymerase, an mRNA capping enzyme, a poly (A) polymerase, and other enzymes required for the synthesis of mRNA during the early stages of infection. Presumably, the unusual shape and structure of the vaccinia virion is the key to its extraordinary biogenesis.

Vaccinia Life Cycle

The vaccinia life cycle, including DNA replication and viral transcription, takes place solely in the cytoplasm of the infected host cell (reviewed in Moss, 2007). Whereas other ds DNA viruses enter the host cell nucleus and use the host machinery in order to replicate, the large genome of vaccinia virus encodes all of the enzymes and factors to carry out its entire replication and transcription in the cytoplasm of the infected cells; this is exceptional for a DNA virus. The vaccinia virus genome comprises three classes of genes: early, intermediate and late, each controlled by its class specific promoters. Viral transcription proceeds in a cascade reaction

where each class of genes codes for factors required for the next class to transcribe. Figure 1-3 demonstrates the major steps of the vaccinia life cycle. The viral particle infects the host cell by fusion of the outer viral membrane with the host cell plasma membrane and releases the core and lateral bodies into the host cell cytoplasm. The viral early transcription factors (VETF) and compliment enzymes that are packaged in the viral core are activated after entry into the host cell and early mRNAs are synthesized and released into the cytoplasm. Early mRNAs code for proteins that are required to dissolve the viral core releasing viral DNA into the cytoplasm, and for replication factors such as DNA polymerase that are required for subsequent DNA replication. DNA replicates in head-to-head and tail-to-tail concatemers, large multimeric genomic molecules. Later during infection, the concatemers resolve into single unit length genomes using the viral resolvase, a late gene product. Besides enzymes for DNA replication, early viral mRNA codes for viral intermediate transcription factors (VITF) that are required for transcription of intermediate genes that in turn code for viral late transcription factors (VLTF). VLTF and viral RNA polymerase transcribe late genes that code for structural proteins required for assembly of a new virus and for VETF and RNA polymerase that is packaged into new viral particles for the next round of infection.

Virus Assembly

Virtually all steps of virus assembly were uncovered using electron microscopy images of cells infected with vaccinia virus. Vaccinia assembly was recently reviewed (Condit et al., 2006). In early times after infection, areas clear of any cellular organelles and sometimes bound by ER appear in the host cell cytoplasm. These cytoplasmic sites are DNA factories, where final assembly of structural proteins and replicated DNA into viral particles take place. Basic steps of vaccinia assembly are shown in Figure 1-3. The first evidence of virus morphogenesis is the appearance of crescents (C), the curved viral membranes containing a lipid bilayer and a D13

scaffold protein. Further, growing crescents morph into three dimensional spheres that ingest surrounding viroplasm, which presumably is genetic material intercalated with protein and evident throughout factories, resulting in immature virions (IV). Simultaneously, IVs with electron dense nucleoids appear, called immature virions with nucleoid (IVN). Serial sections of infected cells suggest that possibly all IVs are contain nucleoids (Morgan et al., 1955). IVN morphs into the mature virion (MV) in a rapid series of events that involve proteolysis of precursor structural proteins, loss of the D13 scaffold protein, internal protein rearrangement, metamorphosis into a brick shape virus with a dense biconcave core, and simultaneous exit of the virion from the factory. Most MVs stay clustered outside of the factories, whereas a small number of MVs continue their morphogenesis by acquiring two additional Golgi derived membranes, becoming wrapped virions (WV) that are ready for exocytosis. WV exits the cell by fusing its outer membrane with the host cell plasma membrane and loosing its outer membrane in order to release the extracellular virion (EV) outside of the cell before spreading within the organism. The vaccinia virus exists in two infectious forms, MV and EV. EV contains two lipid bilayer membranes in contrast to MV, which contains one membrane. MV is a "tough" form of the virus that can exit only by lysing infected cells and is able to spread efficiently between hosts and survive in the outside environment. The additional membrane of EV makes it more sensitive to drying; EV is thought to be important for cell to cell spread within the organism.

Genetic Analysis of Vaccinia Virus

A classic forward genetic approach was applied to study the biology of the vaccinia virus. A collection of temperature sensitive (ts) mutants was generated in a "classic genetic" manner and to this day, serves as a valuable tool to investigate the genetic makeup of vaccinia (reviewed in Condit and Niles, 1990; Kato et al., 2008). The ts mutants are defined as viruses with mutations in essential genes and which grow at a low temperature, 31°C (permissive condition),

but do not grow at a high temperature, 39.7°C (non-permissive condition). The mutants were created by chemical mutagenesis, followed by random screening for temperature sensitivity, complementation analysis and marker rescue analysis that placed them into 38 genes (Condit and Motyczka, 1981; Condit et al., 1983; Dales et al., 1978; Kato et al., 2008; Lackner et al., 2003). Further, mutants in each affected gene were subjected to detailed phenotypic characterization for DNA replication, protein synthesis and assembly of viral particles using electron microscopy. Based on their phenotypes the mutants were placed into three categories: 1) mutants defective in DNA replication; 2) mutants defective in gene expression; and 3) mutants defective in virus morphogenesis.

Preliminary data by Condit and co-workers initially mapped the mutant Cts52 to a region containing genes E2L through E8R (Lackner et al., 2003). The mutant displayed a normal phenotype at both at 31°C and 39.7°C that was indistinguishable from the wild type virus, therefore, the Cts52 was classified in the category of mutants that a play role in virus morphogenesis. Subsequently, the Cts52 mutant was subjected to a more detailed characterization and was mapped to the E6R gene by marker rescue (N. Moussatché, in preparation). The E6R gene function is unknown and mutants in this gene had never characterized. The analysis of Cts52 under non-permissive conditions revealed normal DNA replication and gene expression, and formation of mature virions that were identical to the wild type virion in appearance. However, the mutant virions purified from non-permissive infections were defective in an *in vitro* virion core transcription reaction, affirming the initial hypothesis that Cts52 was defective in morphogenesis.

To continue investigation of the E6R gene, an inducible (ind) mutant, vE6i, provided by P. Turner and R. Moyer, was characterized. The principal difference between ts and ind mutants is

in state of the mutant gene product under the non-permissive condition. Under the permissive condition, both mutants behave the same as the wild type, whereas under the non-permissive condition, a ts mutant may produce a stable but non-functional protein while the ind mutant protein is absent, thus providing an opportunity to examine the vaccinia life cycle in the absence of the E6R gene product. Historically, ts and ind mutants in the same gene may produce radically different phenotypes. Therefore, characterization of the vE6i mutant was genuinely intriguing and further study promises to uncover the E6R gene function.

The E6R Gene

In a mass spectrometry analysis of whole virions, the E6 protein was identified and ranked 13th in abundance among the other seventy proteins that comprise the mature virion (Chung et al., 2006; Yoder et al., 2006). This suggests that the E6 protein is a structural protein of the mature virion, but its exact function is unknown. On a physical gene map of vaccinia, the E6R gene is the sixth gene from the left end of the HindIII E fragment, the "R" indicating "right," which is the direction that the gene is transcribed. The E6R gene maps to a cluster of housekeeping genes on the vaccinia genome, suggesting that the E6R gene itself is also conserved. Figure 1-4 shows a phylogeny tree of the alignments of E6R genes throughout the entire *Poxviridae* family, confirming that the E6 gene is highly conserved; moreover this analysis suggests that E6 is an essential protein. E6 has no homology to any other known proteins outside of the poxvirus family. The E6R coding sequence is 1704 nucleotides in length and translates into a 567 amino acid protein with a molecular weight of 66.6 kilodalton (kDa). Significant insight into E6R gene expression can be gained from examining the E6R gene sequence. Figure 1-5 presents the E6 gene sequence with flanking sequences of adjacent partial E5R and E7R genes on the left and right end respectively. Analysis of the sequence reveals two signatures that suggest that E6R is a post-replicative gene. First, the sequence TAAAA, which is located

immediately upstream of the translation start codon ATG, is a site that is recognized by RNA polymerase and known as a promoter for intermediate and late genes. Second, the T5NT motifs embedded throughout the E6R coding sequence are known as termination transcription signals for early genes, therefore the E6 protein can be expressed only late during infection.

To summarize, E6R is a conserved gene that encodes a late structural protein, which is essential in the formation of a mature virion. The precise function of E6R is still unknown and further investigation is needed.

Aim of Study

Seventy gene products make up a mature viral particle and the mutants in fifty of these genes are available for study. Many of these mutants have been characterized and a gene function revealed. Recent proteomic analysis of the purified vaccinia virion identified E6 protein as a structural protein(Chung et al., 2006; Yoder et al., 2006). Mutants in the E6R gene are available but have not been characterized to date. The aim of this study is to characterize mutants in the E6R gene in order to determine the role of E6 in vaccinia virion structure or assembly.

To understand the role of the E6 gene in viral morphogenesis, two mutants, temperature sensitive mutant Cts52 and inducible mutant vE6i, have been phenotypically characterized. The Cts52 mutant was investigated by A. Strahl and N. Moussatché (N. Moussatché, in preparation). To summarize, Cts52 is a conditionally lethal mutant with a missense mutation in the E6R gene that results in substitution of the 226th amino acid from proline (P) to leucine (L). The mutant grows at 31°C (permissive condition) producing normal E6 protein and lacks growth at 39.7°C (non-permissive condition) producing a nonfunctional E6 protein. The ts mutant displays normal patterns of DNA replication and gene expression, and electron microscopy indicates normal viral morphogenesis at both temperatures. Mutant viral particles purified from infection at the non-

permissive temperature apparently contained the normal complement of virion proteins, including E6, but were non-infectious. The isolated mutant viral core was unable to direct viral mRNA synthesis in vitro. The phenotype of Cts52 suggests that the E6 gene may play a subtle role in virus transcription.

The vE6i mutant is an inducible mutant constructed in the vaccinia vT7lacOI viral expression vector in which the E6 gene is controlled by a *lac* operator and T7 promoter. In vE6i, synthesis of the E6 protein occurs only in presence of inducer, IPTG; without IPTG no E6 protein is produced. Phenotypic analysis of the vE6i mutant will provide a unique opportunity to investigate viral morphogenesis in the absence of E6 protein.

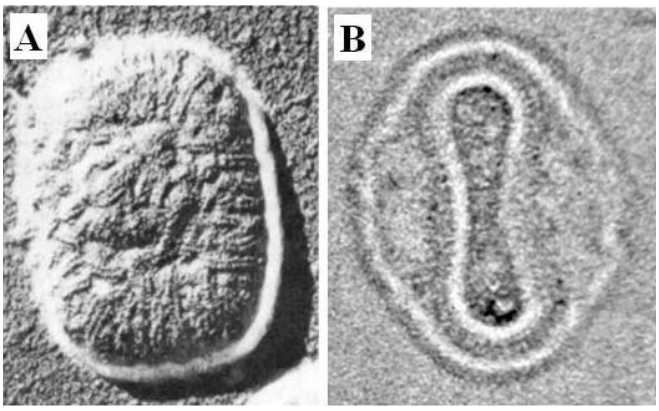

Figure 1-1. Vaccinia virus structure. A) Freeze-etch electron micrograph of virion surface. B) Cryo-section of the virion internal structure. (Reprinted with permission from Condit et al., 2006; figure 1, page 44).

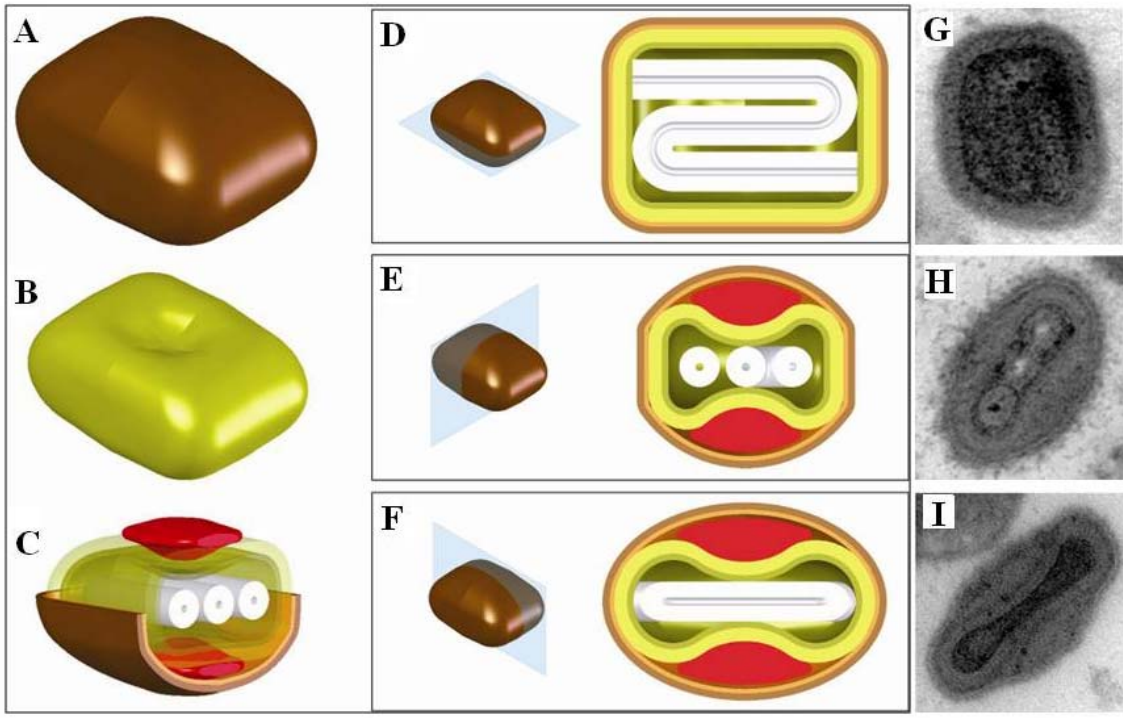

Figure 1-2. Model of vaccinia virus structure. A) The whole virion. B) The virion core. C) The sectioned view displays the virion surface with a lipid bilayer and the internal structure including core with indentations residing lateral bodies and genomic tubules in the center. D-F) The virion sections display internal structure in different planes. G-I) The electron micrographs of viral particle are corresponding to the virion model D-F (Reprinted with permission from Condit et al., 2006; figure 2, page 46; figure 4, page 53).

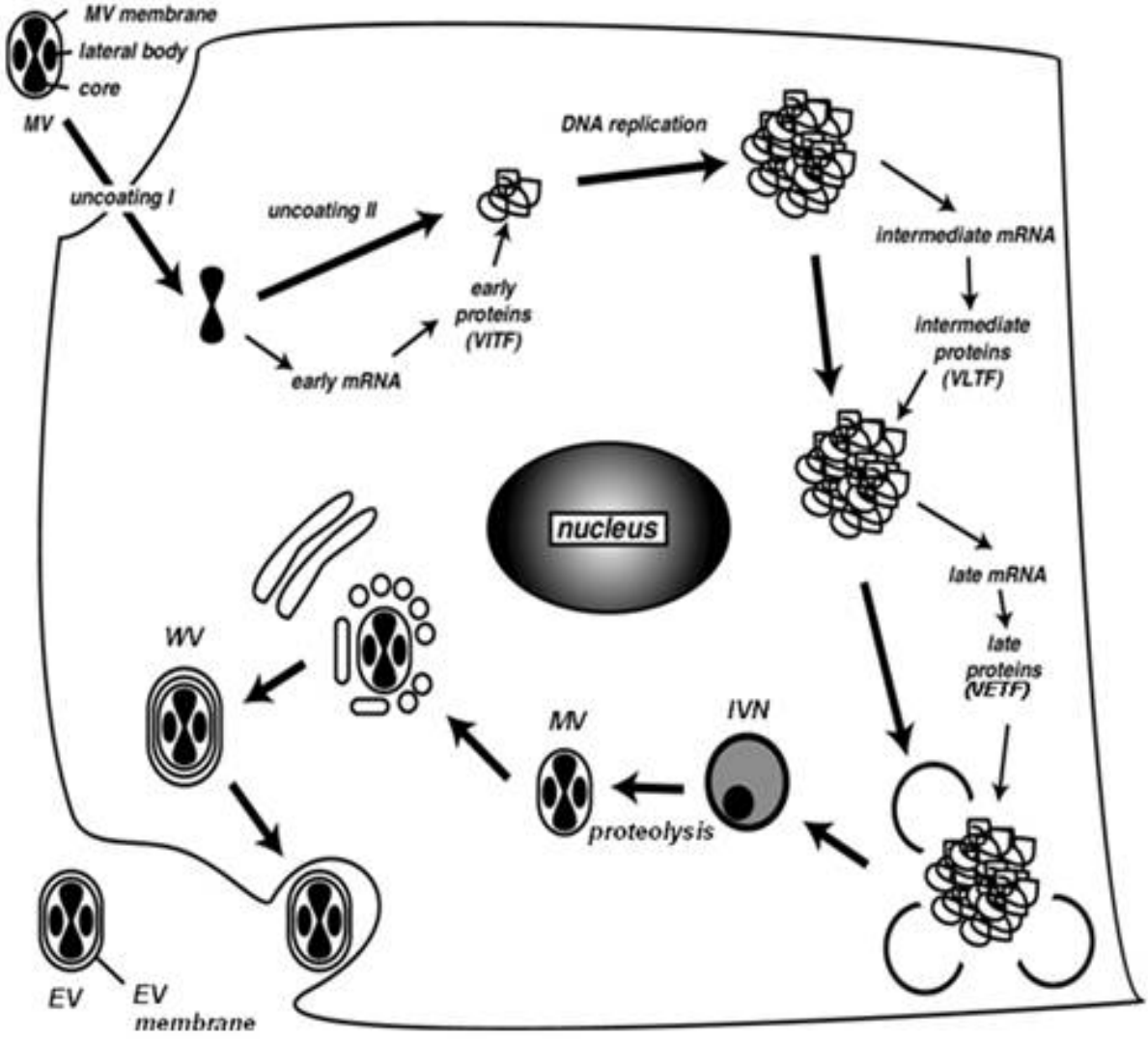

Figure 1-3. Vaccinia virus life cycle. IVN-immature virion with nucleoid; MV-mature virion; WV-wrapped virion; EV-extracellular virion; VITF-viral intermediate transcription factors; VLTF-viral late transcription factors; VETF-viral early transcription factors. See text for additional details. (Courtesy of R. Condit).

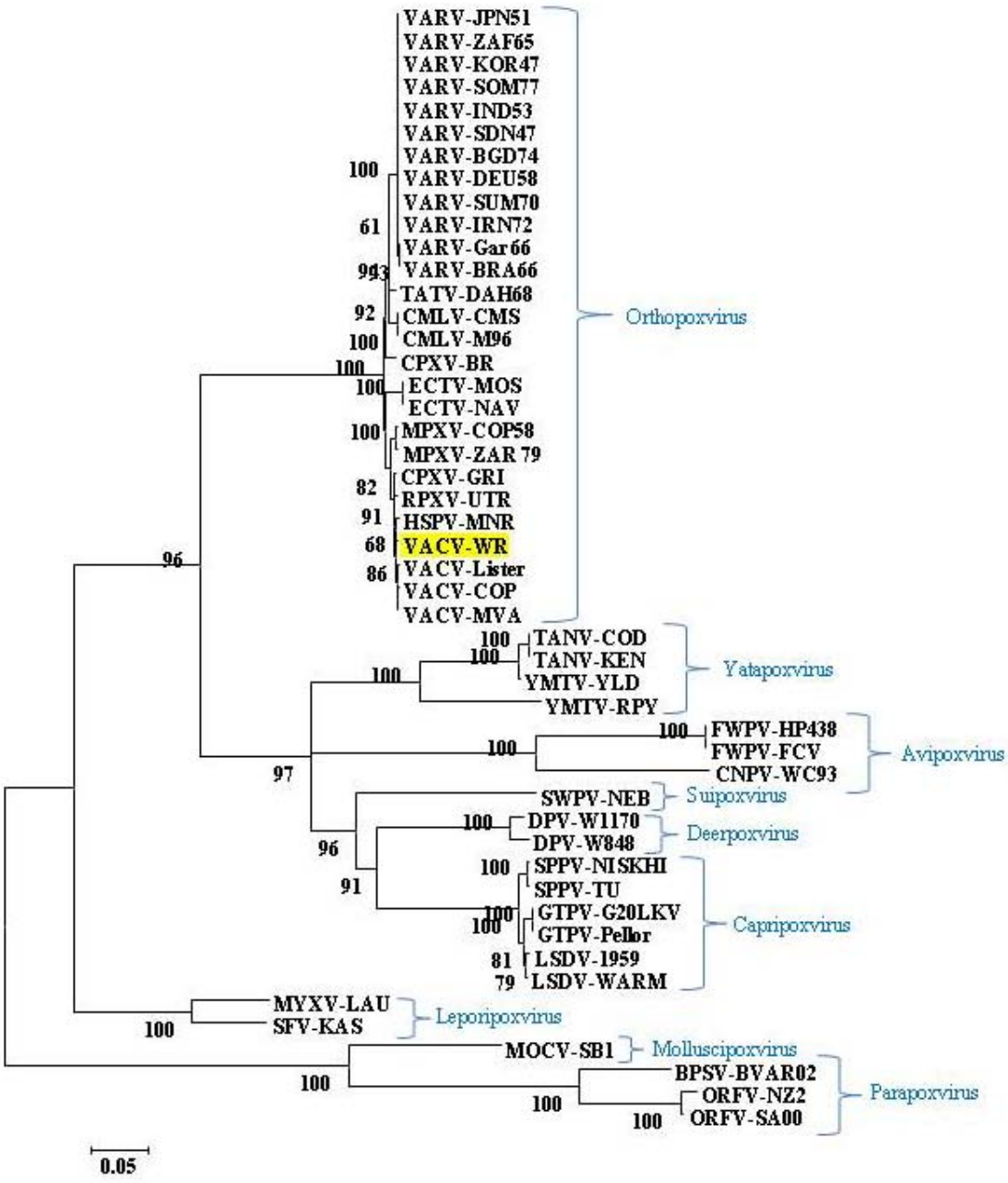

Figure 1-4. The E6 gene phylogeny tree. Neighbor-Joining with pair wise deletion, Kimura 2 model, boot strapped with 1000 replications.

GGCATTTCATCTTTCTCCAATACTAATTCAAATTGTTAAATTAATAATGGATAGTATAAATAGT
TATTAGTGATAAAATAGTAAAAATAATTATTAGAATAAGAGTGTAGTATCATAGATAACTCTCT
TCTA*TAAAA**ATG*GATTTTATTCGTAGAAAGTATCTTATATACACAGTAGAAAATAATATAGATT
TTTTAAAGGATGATACATTAAGTAAAGTAAACAATTTTACCCTCAATCATGTACTAGCTCTCAA
GTATCTAGTTAGCAATTTTCCTCAACATGTTATTACTAAGGATGTATTAGCTAATACCAATTTT
TTTGTTTTCATACATATGGTACGATGTTGTAAAGTGTACGAAGCGGTTTTACGACACGCATTTG
ATGCACCCACGTTGTACGTTAAAGCATTGACTAAGAATTATTTATCGTTTAGTAACACAATACA
ATCGTACAAGGAAACCGTGCATAAACTAACACAAGATGAAAAATTTTTAGAGGTTGCCAAATAC
ATGGACGAATTAGGAGAACTTATAGGCGTAAATTATGACTTAGTTCTTAATCCATTATTTCACG
GAGGGGAACCCATCAAAGATATGGAAATCATTTTTTTAAAACTGTTTAAGAAAACAGACTTCAA
AGTTGTTAAAAAATTAAGTGTTATAAGATTACTTATTTGGGCTTACCTAAGCAAGAAAGATACA
GGCATAGAGTTTGCGGATAATGATAGACAAGATATATATACTCTATTTCAACAAACTGGTAGAA
TCGTCCATAGCAATCTAACAGAAACGTTTAGAGATTATATCTTTCCCGGAGATAAGACTAGCTA
TTGGGTGTGGTTAAACGAAAGTATAGCTAATGATGCGGATATTGTTCTTAATAGACACGCCATT
ACCATGTATGATAAAATTCTTAGTTATATATACTCTGAGATAAAACAGGGACGCGTTAATAAAA
ACATGCTTAAGTTAGTTTATATCTTTGAGCCTGAAAAAGATATCAGAGAACTTCTGCTAGAAAT
CATATATGATATTCCTGGAGATATCCTATCTATTATTGATGCAAAAAACGACGATTGGAAAAAA
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TCCAAGAGTAGACGGCTGAACTTGTTTAGCAAAAACATTTTAAAATATTATTTAGACGGACAAT
TGGCTCGTCTAGGTCTTGTGTTAGATGATTATAAAGGAGACTTGTTAGTTAAAATGATAAACCA
TCTTAAGTCTGTGGAGGATGTATCCGCATTCGTTCGATTTTCTACAGATAAAAACCCTAGTATT
CTTCCATCGCTAATCAAAACTATTTTAGCTAGTTATAATATTTCCATCATCGTCTTATTTCAAA
GGTTTTTAAGAGATAATCTATATCATGTAGAAGAATTCTTGGATAAAAGCATCCATCTAACCAA
GACGGATAAGAAATATATACTTCAATTGATAAGACACGGTAGATCA*TAG*AACAGACCAAATATA
TTATTAATAATTTGTATATACATAGATATAATTATCACATATTAAAAATTCACACATTTTTGAT
AA

Figure 1-5. The E6R gene sequence. The E6R coding region is underlined. Partial flanking sequences of the E5R and E7R genes on the left and right, respectively, are shown. Translation initiation and termination codons of the E6 gene are shown in blue. The TAAAA sequence (shown in red) located just upstream of the ATG start codon is a promoter for intermediate and late genes. T5NT early transcription termination motifs within the E6 coding region are shown in red.

CHAPTER 2
MATERIALS AND METHODS

Cells

All experiments were performed using confluent monolayers of BSC40 cells, a continuous cell line from African green monkey kidney. Cells were maintained in Dulbecco modified Eagle medium (DME) supplemented with 10% fetal bovine serum (FBS, Hyclone) at 37°C in a 5% CO_2 incubator.

Viruses and Plasmids

Stocks of the wild type (wt) vaccinia virus (strain WR) and the temperature-sensitive (ts) mutant virus Cts53 were used in infections as controls; conditions for virus growth, infections, and virus cultures were previously described (Condit and Motyczka, 1981; Condit et al., 1983). The permissive temperature for the ts mutant is 31°C and the non-permissive temperature is 39.7°C. The inducible (ind) mutant vE6i was constructed by P. Turner and R. Moyer and generously provided to us for our investigation. vE6i uses the vT7lacOI vaccinia expression vector (Alexander et al., 1992) with the E6R gene expression under control of the T7 RNA polymerase promoter and *Escherichia coli* (*E. coli*) lactose (*lac*) repressor and *lac* operator, described in more detail in the results section. The vE6i mutant growth and infections were carried in the presence of 50 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). pBD6 is a recombinant plasmid containing 2.6 kb *Bst*EII concatemer junction of vaccinia virus cloned in the pUC13 bacterial expression plasmid and provided by M. Merchlinsky (Merchlinsky and Moss, 1989).

Plaque Assay

Confluent 60mm dishes of BSC40 monolayers were infected with wt and vE6i in 10-fold serial dilutions in PBS-AM (PBS + 1% Albumin +10 mM $MgCl_2$). Following 30 min of

absorption, cells were overlaid with plaque assay media (1 volume 2 X DME + 10%FBS + 1 volume 2% methyl cellulose – or + IPTG). After 4 days of incubation at 37°C, dishes were stained with crystal violet (0.26% crystal violet; 10% ethanol; ~22% formaldehyde) to quantify plaques.

One-Step Growth Assay

Confluent 35 mm dishes of BSC40 cells were infected with wild type and vE6i viruses at a multiplicity of infection (moi) of 10 plaque-forming units per cell (pfu/cell) and incubated for 30 minutes at 37°C. After absorption, the cells were washed twice with PBS-AM, then DME containing or lacking IPTG was added, and the infected cells were incubated at 37°C. At 0, 3, 6, 9, 12, 24 hours post infection (hpi), the cells were harvested and viral titers measured by plaque assay.

Slot-Blot DNA Analysis

35mm dishes of confluent BSC40 cells were infected at a moi of 10 pfu/cell as described above. At different times after infection, the infected cells were harvested in loading buffer (10 X SSC; 1 M NH_4OAc) and the samples were prepared as described (Damaso et al., 2002). The cell samples were applied in triplicate on an uncharged nylon membrane (GeneScreen), which was assembled in a Slot Blot Minifold II apparatus (Schleicher and Schuell, Inc.). The DNA samples were denatured *in situ* with a denaturation solution (1.5 M NaCl /0.5 N NaOH; 10 X SSC) and neutralized with 10 X SSC solution. The membrane was then removed from the slot blot apparatus and hybridized with a linearized vaccinia HindIII E fragment probe [^{32}P]-labeled using a DECAprime II-Random Primed DNA Labeling Kit (Ambion) following the manufacturer's protocol. Hybridization was done in a solution containing 6 X SSC, 50 % formamide, 0.5 % SDS, 5 X Denhardts, 100 µg/ml denatured salmon sperm DNA at 42°C overnight. The blot was washed once for 20 minutes at room temperature and twice for 20

minutes at 50°C with wash buffer (6 X SSC, 0.1% SDS, 0.05% $Na_4P_2O_7$). The blot was analyzed using a phosphor-imager (Storm 860, GE-Healthcare).

Metabolic Protein Labeling

For pulse-labeling, the cells were infected at moi of 10 pfu/cell as described above. At time intervals 0,3,6,9 hpi, [^{35}S]-labeled methionine (Perkin Elmer) in methionine free medium 50 μCi/dish was added, followed by 30 minutes of incubation. After incubation, the medium was removed and cells were lysed in a Laemmli sample buffer (50 mM Tris-HCl, pH6.8; 1% SDS; 1% glycerol; 100 mM β-mercaptoethanol; 0.1% bromophenol blue) and stored at -80°C.

For pulse-chase, at 9 hpi, [^{35}S]-labeled methionine in methionine free media was added for a 30 minute incubation as described above, and then replaced with media supplemented 1 mM unlabelled methionine. Cells were harvested in Laemmli buffer at 12 and 24 hpi. Metabolically labeled proteins for pulse-labeling and pulse-chase were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by autoradiography (Kato et al., 2004).

Electron Microscopy

For transmission electron microscopy (TEM), 60 mm dishes of BSC40 cell monolayers infected with 10 pfu/cell of wild type and vE6i mutant for 24 hr and 48 hr in the presence of absence of IPTG. At the designated times, cell monolayers were rinsed with 0.1 M sodium cacodylate buffer, pH 7.4 and fixed with 2 % glutaraldehyde in 0.1 M cacodylate buffer containing 2 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.25 % NaCl, pH 7.24 (provided by ICBR). Prepared samples were submitted for TEM analysis to the ICBR Electron Microscopy Core Laboratory of University of Florida.

Southern Blot

Confluent 100 mm dishes of BSC40 cells were infected with wild type virus and mutants Cts53 and vE6i as previously described. At 6, 12, and 24 hpi, cells were scraped from the dishes and DNA isolated using a DNeasy tissue kit (Qiagen) following the manufacturer's protocol. Isolated DNA was digested with *Bst*EII (Promega) for 20 hours at 37°C, as described (Damaso et al., 2002). Digested DNA fragments were separated on a 1 % agarose gel in TAE buffer, denatured in1.5 M NaCl/0.5 M NaOH, neutralized in 1.5 M NaCl/0.5 M Tris-Cl, pH 7.0 and transfered to an uncharged nylon membrane (GeneScreen) by Southern blotting using 20 X SSC transfer buffer. The membrane was hybridized as described above with a [^{32}P]-labeled pBD6 probe and analyzed using a phosphor-imager (Storm 860, GE-Healthcare).

CHAPTER 3
RESULTS

The vE6i Construct

To investigate the role of the E6R gene in vaccinia morphogenesis, the inducible mutant vE6i constructed by P. Turner and R. Moyer was provided for phenotypic characterization. Previous published work indicated that a difference in the phenotype sometimes exists between ts and ind mutants (Mercer and Traktman, 2005). This difference theoretically results from the difference in mechanism of conditional lethality of the mutants under the non-permissive condition. In the ts mutant, the target protein may be stably expressed but nevertheless be nonfunctional, whereas in the ind mutant the target protein is not expressed at all. Therefore, the analysis of the inducible mutant phenotype provides an opportunity to evaluate how the absence of the E6 protein affects vaccinia biogenesis.

The recombinant virus vE6i was constructed using the vT7lacOI vaccinia virus vector with expression of the E6 protein controlled by a *lac* operator/repressor (*lacO/I*) system and regulated by the inducer IPTG. As shown in Figure 3-1, the construct comprises two parts: 1) prokaryotic *lac* operon transcriptional regulatory elements positioned in the thymidine kinase (TK) locus of vaccinia and 2) replacement of the native E6R promoter with *lac* operator-regulated prokaryotic transcriptional elements. In detail, the TK locus of the construct contains the gene encoding T7 RNA polymerase, which is under control of the vaccinia late promoter P11with a *lacO* positioned in between. This is followed with the *lacI* gene placed under control of the constitutively active vaccinia early/late promoter P7.5. Within the endogenous E6R locus, E6R gene is placed under control of the bacteriophage T7 RNA polymerase transcriptional promoter and a *lacO* positioned in between. Also, the construct includes the dominant selectable marker

E. coli guanine phosphoribosyltransferase (gpt) under control of the early/late P7.5 vaccinia transcriptional promoter, crucial only during selection of a correct construct.

The construct is highly efficient by achieving 99% in suppression of the E6 protein synthesis. The effectiveness build in the vE6i mode of action is as follows: in absence of inducer IPTG (non-permissive condition), the constitutively expressed *lacI* gene encodes repressors that bind to the *lacO* and prevents transcription of the gene encoding T7 RNA polymerase and simultaneously suppresses expression of the E6 protein. In the presence of an inducer (permissive condition), IPTG binds to active repressors and induces their conformational change, which disable binding of repressors to the *lacO*. The transcriptional complex can assemble and the T7 gene encodes T7 RNA polymerase, which binds to the T7 promoter and transcribes the E6 gene, thereby synthesizing the E6 protein.

The vE6i mutant Confirms its Inducible Phenotype

The initial step in phenotypic characterization of the vE6i mutant was to evaluate the mutant's basic growth properties by plaque assay. This analysis answers the fundamental questions of: 1) whether the vE6i mutant was able to grow and form plaques and 2) whether the mutant was repressible, so that morphogenesis could be studied in the absence of the E6 protein. Accordingly, confluent monolayers of BSC40 cells were infected with vE6i in 10-fold serial dilutions, overlayed with plaque medium in the presence or absence of IPTG. After 4 days of incubation, the plaques were visualized (Figure 3-2). In the presence of the IPTG, vE6i formed plaques throughout the 10^{-7} dilution, which demonstrates the mutant's ability to produce infectious viral particles in a fashion equivalent to wild type virus. In the absense of inducer, the plaques were evident only at the 10^{-3} dilution proving that the mutant is inducible. Wild type virus formed plaques with equal efficiency in the presence and absence of IPTG (Figure 3-3). The plaques formed by vE6i at low dilution in the absence of IPTG are presumably revertants.

Spontaneous reversion could involve mutational inactivation of the *lacI* which thus allows expression of the E6R gene in the absence of inducer. The reversion rate is 1 in 30,000, which is not sufficiently significant to distort the results of the phenotypic characterization of the mutant. It is noteworthy that under the permissive condition vE6i produces plaques that are smaller in size compare to wild type (Figure 3-3). In an attempt to increase the plaque size of vE6i, we tested IPTG at a variety of concentrations: 25, 50, 75, and 100 μM. The results showed that at 25 μM IPTG there was a 50 % decrease in plaques size (Figure 3-3) and count (data not shown) compared to 50 μM; there is no difference in the plaque size or quantity using IPTG concentrations above 50 μM. Thus, the use of the 50 μM concentration was continued. Noteworthy mentioning that many inducible constructs are form significantly smaller plaques compare to wild type that could be due to reduced virus spread to nearby cells. Based on the plaque assay, it was concluded that the vE6i mutant has a tightly inducible phenotype; consequently it is a reliable mutant candidate for study of the E6R gene.

The E6 Protein is Essential in Virus Biogenesis

To quantitatively evaluate whether the E6 protein is essential in the vaccinia viral cycle, the vE6i mutant was analyzed using a one-step growth assay. The ultimate goal of this assay is to investigate the time course of virus replication in the absence of the E6 protein. Confluent cell monolayers were infected with wild type virus or the vE6i mutant at a moi of 10 pfu/cell and grown in the presence of absence of IPTG. At various times, the cells were harvested and viral titers measured by plaque assay. Figure 3-4 displays graphs of viral growth curves for each virus under the permissive and non-permissive conditions. Wild type infection under the permissive and non-permissive condition has the same pattern: in the first hours after infection the virus attaches to the cells, follow by eclipse in the growth curve by 3 hpi, where the virus enters the cells, uncoats, and viral infectivity is lost, followed by rapid virus growth where the virus

replicates, producing by 24 hpi, 34 and 38 particles per cell respectively. On the graph for the vE6i mutant, the growth pattern in the presence of IPTG is similar to the wild type overall, though lagging in virus growth up to 6 hpi, it then catches up by 24 hpi producing 50 particles per cell. In the absence of IPTG, replication of the vE6i mutant is significantly reduced forming approximately 0.2 infectious particles per cell. The fact that the vE6i mutant failed to replicate at the non-permissive condition, confirms that the E6 protein is vital for the virus life cycle.

The vE6i mutant is Normal in DNA Replication

To uncover which step of viral growth is affected by the E6R gene, DNA synthesis was measured in a vE6i infection done under permissive and non-permissive conditions. Cells were infected with wild type and vE6i in the presence and absence of IPTG. At designated times, cells were harvested, DNA isolated and accumulation of DNA measured in a slot blot protocol by hybridization to a radioactively labeled vaccinia DNA probe (Materials and Methods). A plot of DNA replication during wt and vE6i infections in the presence or absence of IPTG is shown in Figure 3-5. The curves of all four infections exhibit identical DNA synthesis pattern with steep growth launched at early hours after infection reaching a maximum at 12 hpi and followed by a plateau. Therefore the vE6i mutant is not defective in DNA synthesis showing that the E6R gene is not essential for vaccinia DNA replication.

The vE6i mutant is Normal in Protein Synthesis; but Defective in Proteolysis

Since the vE6i mutant is not defective in DNA replication, the next step was to examine vE6i in protein synthesis and proteolysis of the major virion structural precursor proteins, p4a and p4b, which is required for the transition during virus assembly from immature to mature virions. Accordingly, cells were infected with wt and vE6i viruses under permissive and non-permissive conditions. At designated times, the infected cells were pulsed with radioactively labeled methionine, the cells were harvested, and cell extracts were separated by gel

electrophoresis and visualized by autoradiography (Figure 3-6). At early hours, host cell protein synthesis is apparent, then it shuts off by 3 hpi and early viral protein synthesis takes place. By 6 hpi, late proteins were synthesized and persisted throughout infection including precursor proteins p4a and p4b. Analysis of metabolically labeled proteins in wt and vE6i infections suggested that protein synthesis during vE6i infection is equivalent to the wild type infection.

Further, vE6i was examined for proteolytic processing of precursor proteins p4a and p4b. Proteolysis in vaccinia virus is represented by a cleavage of precursor proteins p4a (102 kDa) and p4b (73 kDa) into the final product proteins 4a (62 kDa) and 4b (60 kDa) respectively (Kato et al., 2004). Proteolytic processing serves as a major indicator of the transition from immature to mature virions. Cells infected with wt and vE6i under permissive and non-permissive conditions were metabolically labeled at 9 hpi, chased, and then harvested at 12 and 24 hpi. Proteins were separated by gel electrophoresis and visualized by autoradiography. The same pattern was evident in the wild type infection in the presence or absence of the inducer and in the vE6i infection in presence of inducer. At 9 hpi, the precursor proteins p4a and p4b appeared on the gel as bands of 102 and 73 kDa. At 12 and 24 hpi, a reduction in the intensity of the precursor bands at 102 kDa and 73 kDa was observed and at the same time, additional bands 62 kDa and 60 kDa appeared that were the cleaved 4a and 4b products respectively. In the vE6i infection without IPTG, the intensity of the bands of precursor proteins was unchanged at 12 and 24 hpi and no additional bands were detectible. Thus proteolysis of virion structural precursor proteins is defective in the vE6i infection under non-permissive conditions. Failure in the proteolysis of the major precursor proteins indicates that vE6i is defective in morphogenesis.

The vE6i mutant Fails to Form Mature Virions

Late during infection, all steps of virus morphogenesis in the host cell cytoplasm can be observed using electron microscopy. The fact that the vE6i mutant is defective in proteolysis

theoretically predicts incomplete viral morphogenesis during vE6i infection. In contrast, analysis of the temperature sensitive mutant Cts52 confirmed that proteolysis took place and mature virions were assembled (A. Strahl and N. Moussatche, personal communication). Therefore, examining EMs and visually comparing the morphogenesis of Cts52 and vE6i mutants could provide insight into E6R gene function. Thus, cells infected with the vE6i mutant under permissive and non-permissive conditions were fixed at 24 hpi and 48 hpi and submitted to ICBR for EM analysis. Electron micrographs of cells infected with wild type virus or with Cts52 at 31°C and 40°C were available for examination as controls. Figure 3-7A is an electron micrograph of the wild type infection at 40°C (non-permissive for Cts52 infection) fixed at 24 hpi and serves as a control for EMs with both mutants. All stages of vaccinia virus morphogenesis were observed in infection with wild type virus including crescents (C), immature virions (IV), immature virion with nucleoid (IVN) and mature virions (MV). EMs with the Cts52 mutant at the permissive temperature at 24 hpi (Figure 3-7B and D) and the non-permissive temperature at 24 hpi (Figure 3-7E) displayed viral assembly indistinguishable from the wild type. In Figure 3-8A and B, infection with vE6i under permissive conditions exhibited normal viral morphogenesis with mature virions clustered in the host cell cytoplasm. By contrast, the infection with vE6i under non-permissive conditions (Figure 3-8C, D and F) revealed aberrant morphogenesis: only empty IVs were present; filled IVs, IVNs and MVs were absent. In addition, large dense crystalloids localized in the periphery of the DNA factories were observed in the cytoplasm. These findings suggested that morphogenesis is arrested prior to formation of normal IV and are consistent with vE6i defective proteolysis, which is critical in the formation of mature virions.

Genomic Concatemers are Resolved in vE6i Infected Cells

The crystalloids and empty IVs observed in the cytoplasm of vE6i infected cells under non-permissive condition were a sign that replicated DNA was accumulated in large aggregates instead of packaged into IVs. Vaccinia DNA replicates in multi-genome concatemers in the early stages of vaccinia morphogenesis (Moyer and Graves, 1981). In the late stage of infection, concatemers are resolved into single genomes that can be packaged into viral particles (Merchlinsky and Moss, 1989) (Figure 3-9). Therefore, the occurrence of crystalloids could be due to accumulation of unresolved DNA. To test this hypothesis, vE6i was subjected to analysis for concatemer resolution. DNA was isolated from cells infected with the wild type, Cts53, and vE6i mutant under permissive and non-permissive conditions. Cts53 is a temperature sensitive mutant defective in the concatemer resolution at 40°C and served as a control (Merchlinsky and Moss, 1989). Isolated DNA was digested with the *Bst*EII restriction enzyme, separated by gel electrophoresis and transferred to a membrane by Southern blotting. The blot was hybridized with a DNA probe containing the 2.6 kb vaccinia concatemer junction and then was visualized by autoradiography. If concatemers are resolved, monomeric genome ends will yield a 1.3 kb fragment whereas unresolved junctions will yield 2.6 kb fragments (Figure 3-10). The analysis of the vE6i infection showed that prominent bands of 1.3 kb are evident in permissive and non-permissive conditions confirming that concatemers were resolved analogous to the wild type infection (Figure 3-11).

Mature Virions Formed after Inducer Added in Blocked Morphogenesis

Electron microscopy revealed that in the absence of IPTG, vE6i failed to form MVs; instead empty IVs and large dense crystalloids were observed in the cytoplasm of infected cells. To understand the nature of the crystalloids, two questions were asked: 1) if permissive conditions are introduced to infections which were initiated under non-permissive conditions and

therefore blocked in morphogenesis, can the viral assembly complete and form mature virions?; 2) if crystalloids are composed of replicated DNA, can this nucleic acid be packaged to assemble new virus? To address these questions, cells were infected with vE6i under the non-permissive condition for 24 hours to induce formation of crystalloids. Subsequently, one set of cells was incubated in the presence of IPTG, another set of cells was incubated in the presence of IPTG and the DNA replication inhibitor cytosine arabinoside (CAR), and another set of cells was incubated in the absence of IPTG as a control. Infected cells were harvested at designated times and viral titers were measured by plaque assay. The results show that when inducer alone or inducer plus CAR were added to infections which had been blocked in morphogenesis, new virus was synthesized producing 0.36 and 0.32 particles per cell respectively. The vE6i control infection in the absence of the inducer showed no change with 0.07 particles per cell produced (Figure 3-12). The synthesis of new virus after addition of IPTG indicates that arrested morphogenesis is reversible. The fact that synthesis of the same amount of new viral particles was produced in the presence of absence of CAR suggests that new virus formed exclusively from existing DNA since CAR inhibits *de novo* DNA replication. This preliminary data provide stronger evidence in support of the hypothesis that formed crystalloids consist of replicated DNA which under permissive conditions can be processed and packaged into new viral particles. To further assess the crystalloids as a source of DNA for new virus synthesis, the experiment described here should be repeated simultaneously with electron microscopy of crystalloids in absence of IPTG and after addition of IPTG and IPTG plus CAR. The EMs will provide visual evidence for processing of crystalloids after addition of IPTG in order to make new virus and completely reverse blocked morphogenesis.

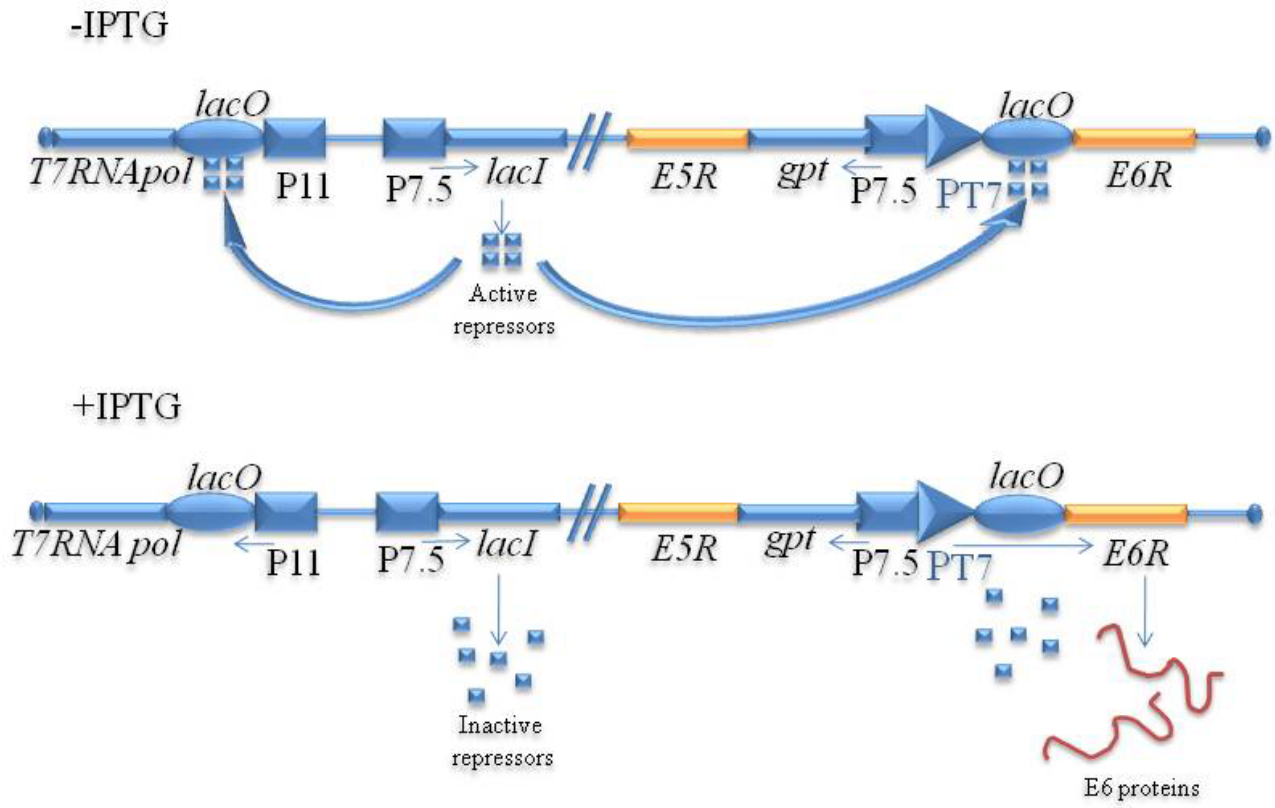

Figure 3-1. The vE6i construct. Scheme of the functional mechanism of the vE6i construct. Under the non-permissive condition (-IPTG), the *lac* repressor (*lacI*) synthesizes active repressors that bind to the *lac* operator (*lacO*) inhibiting both T7 RNA polymerase and E6R gene transcription. Under the permissive condition (+IPTG), the inducer binds to repressors and inactivates them. The T7 RNA polymerase gene transcribed from the vaccinia late promoter P11, synthesizes T7 RNA polymerase that binds to the T7 promoter and transcribes the E6R gene.

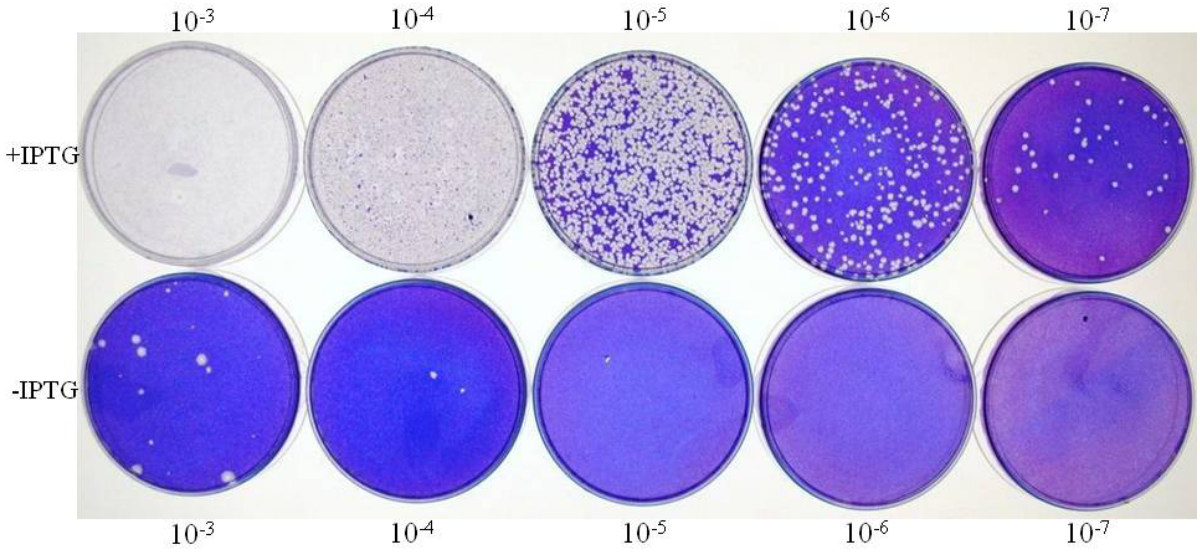

Figure 3-2. Plaque assay of vE6i in presence or absence of IPTG. BSC40 cells infected with vE6i in 10-fold dilutions as indicated and grown in presence or absence of IPTG for 4 days, followed by crystal violet staining.

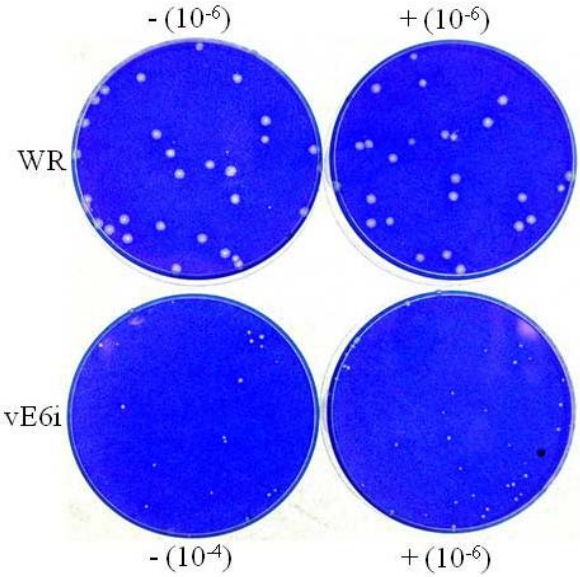

Figure 3-3. Comparison of plaque morphology for cells infected with wild type vaccinia and the vE6i mutant. BSC40 cells were infected with wild type WR and vE6i in 10-fold dilutions and grown in presence or absence of IPTG for 4 days, followed by crystal violet staining. The wild type forms normal size plaques in presence or absence of IPTG, while the small plaque size was observed in vE6i infection with or without IPTG.

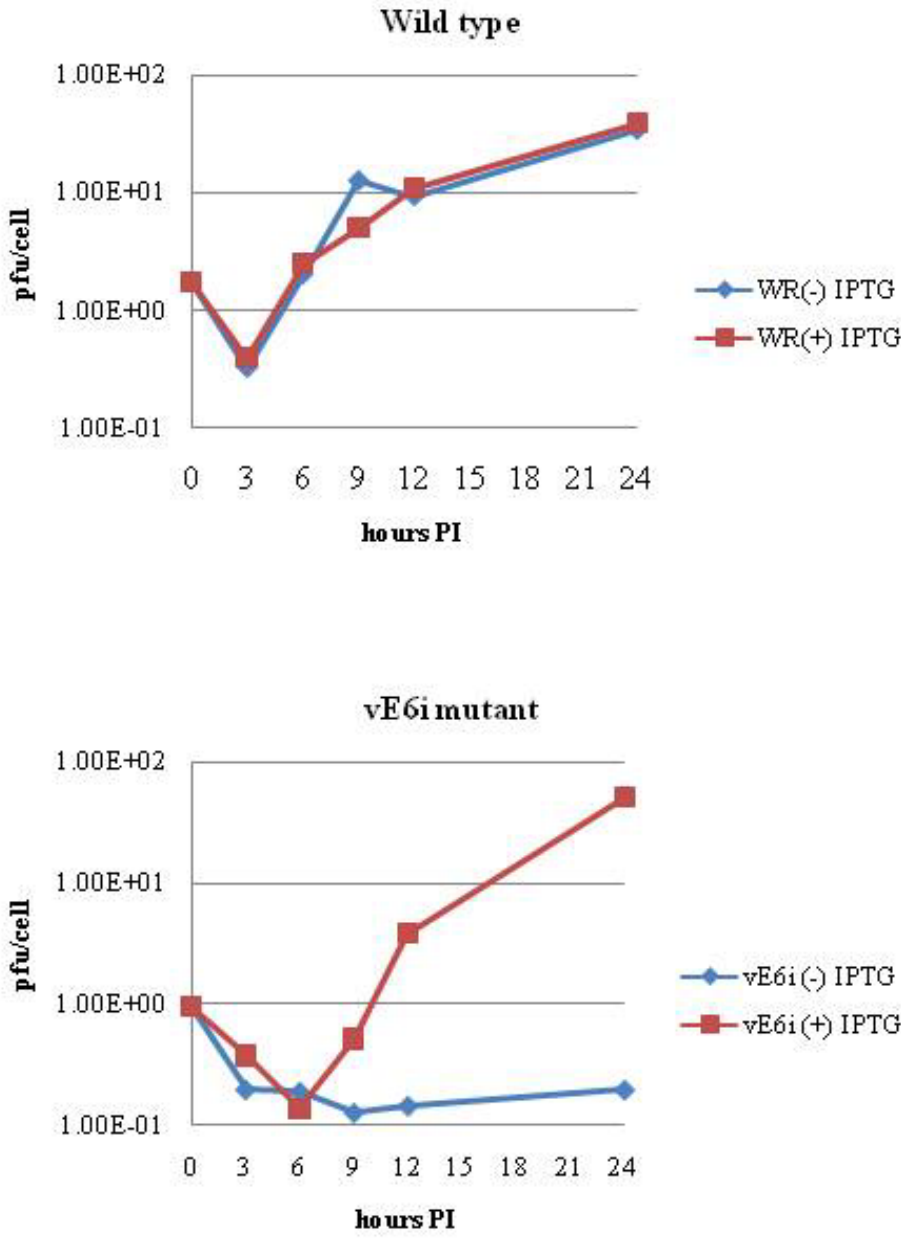

Figure 3-4. One-step growth assay of the wild type and vE6i viruses. BSC40 cells infected with 10 pfu/cell of wt and vE6i viruses in the presence or absence of IPTG, harvested at 0, 3, 6, 9, 12, 24 hour post infection (hpi) and virus titers measured by plaque assay. The X-axis presents hours post infection at which virus was harvested and the Y-axis displays the number of viral particles per cell produced at the designated time.

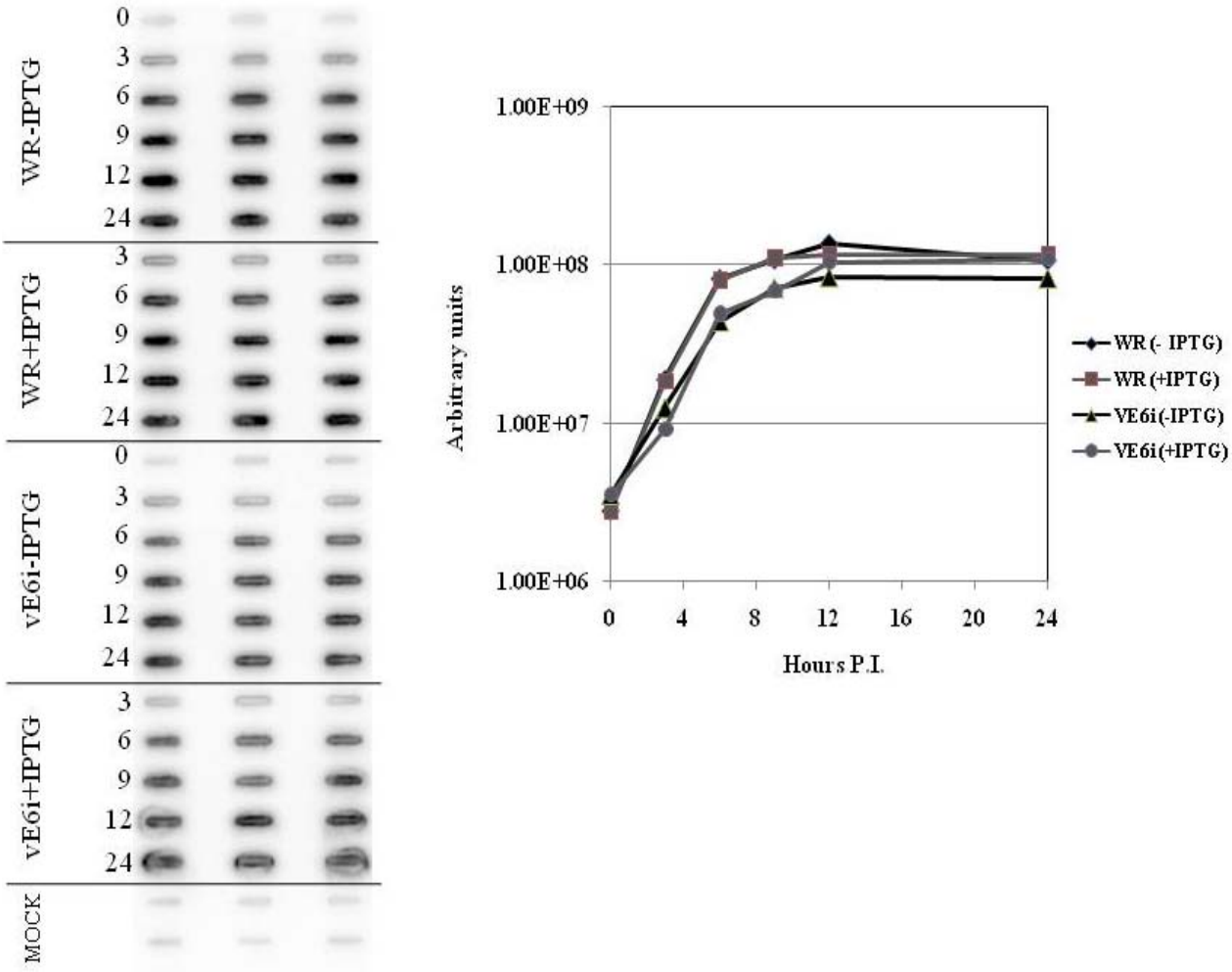

Figure 3-5. The DNA replication. BSC40 cells were infected with 10 pfu/cell of wild type WR and vE6i viruses, harvested at designated times, and hybridized with a radiolabeled vaccinia DNA probe (Materials and Methods). On the left is a phosphorimage of the hybridized slot-blot. The three columns are replicate samples. The last two rows are samples of uninfected cells used as a negative control. On the right is a graph of the phosphorimage data. The X-axis and Y-axis represent hours post infection and arbitrary units from the phosphor-imager, respectively.

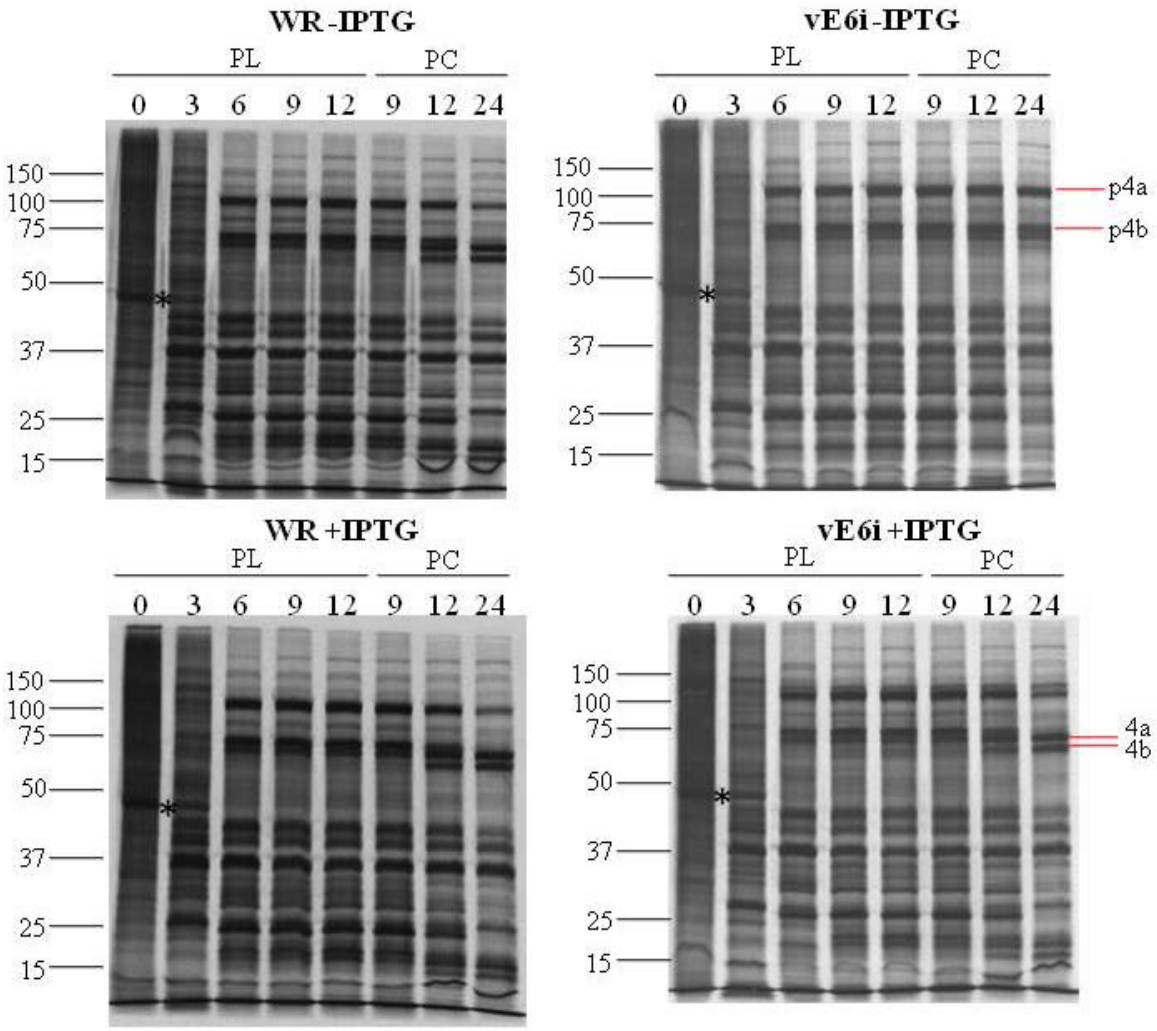

Figure 3-6. Protein synthesis and protein processing in infections with wild type WR and vE6i mutant in the presence and absence of IPTG. PL, pulse-labeling; PC, pulse-chase; *, host proteins. Red lines indicate proteins p4a and p4b before proteolytic processing with MW of 102 kDa and 73 kDa respectively; proteins 4a and 4b after processing have MW of 62 and 60 kDa respectively. Above each autoradiogram the hours of post infection are indicated. At the left of each autoradiogram are indicated the migration of protein standards with molecular weights in kDa. For protein labeling (PL), infected cells were pulsed with [^{35}S]-methionine for 30 min and harvested. For pulse-chase (PC), at 9 hpi [^{35}S]-methionine was added for 30 min then removed, replaced with unlabeled methionine supplemented media, and cells were harvested at 12 and 24 hpi. In PL and PC, the cells lysates were examined by SDS-PAGE and autoradiography.

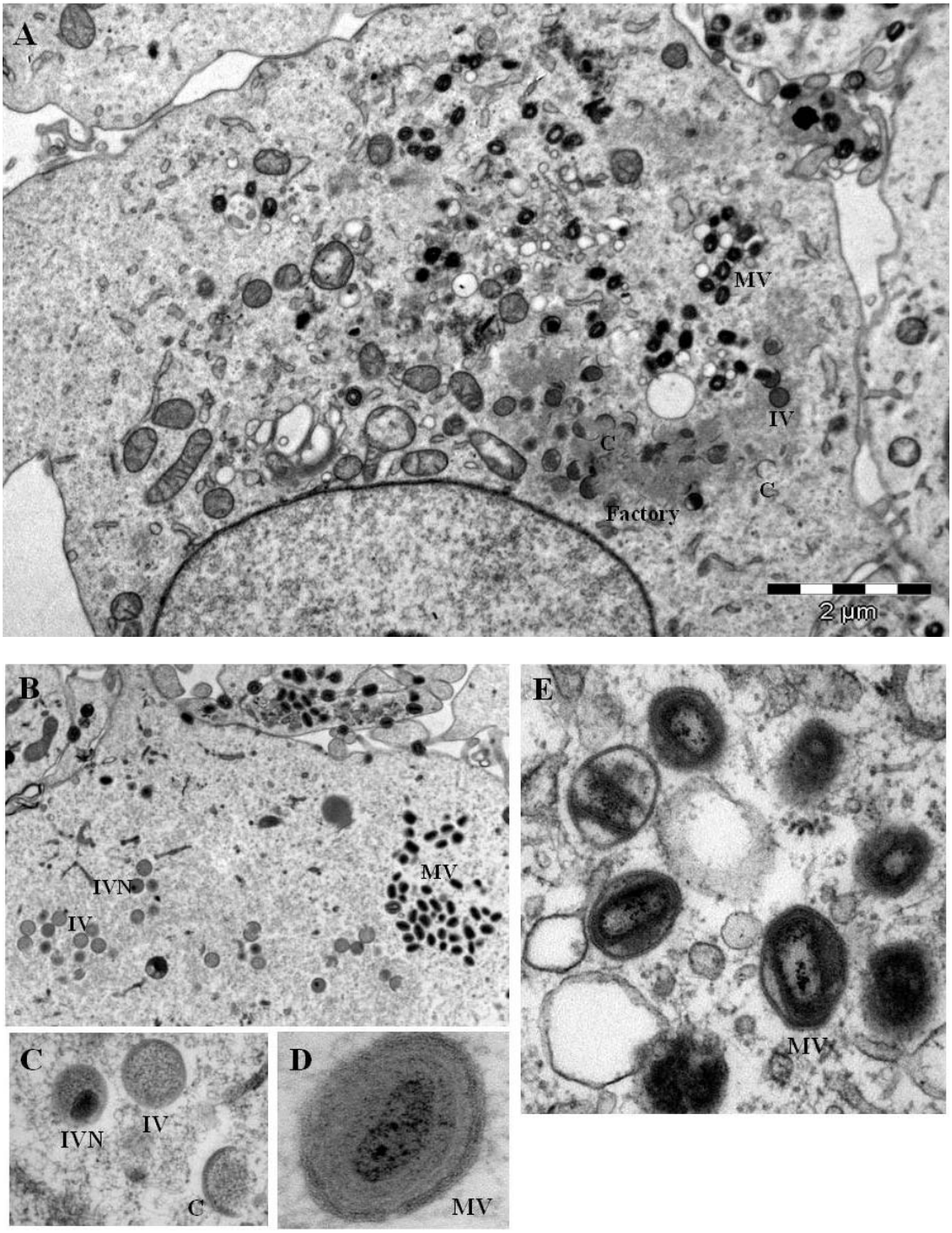

Figure 3-7. Electron microscopy of the wild type and the Cts52 mutant infections. F, DNA factories; C, crescents; IV, immature virions; IVN, immature virions with nucleoids; MV, mature virions. A) EM of wt virus infection for 24 hr at 40°C used as a control. All stages of virus morphogenesis were observed. (EM courtesy of Dr. Sayuri Kato.) B-D) Infection with Cts52 grown at 31°C 24 hr. E) Cts52 at 40°C, 24 hr. (EM courtesy of Audra Strahl.).

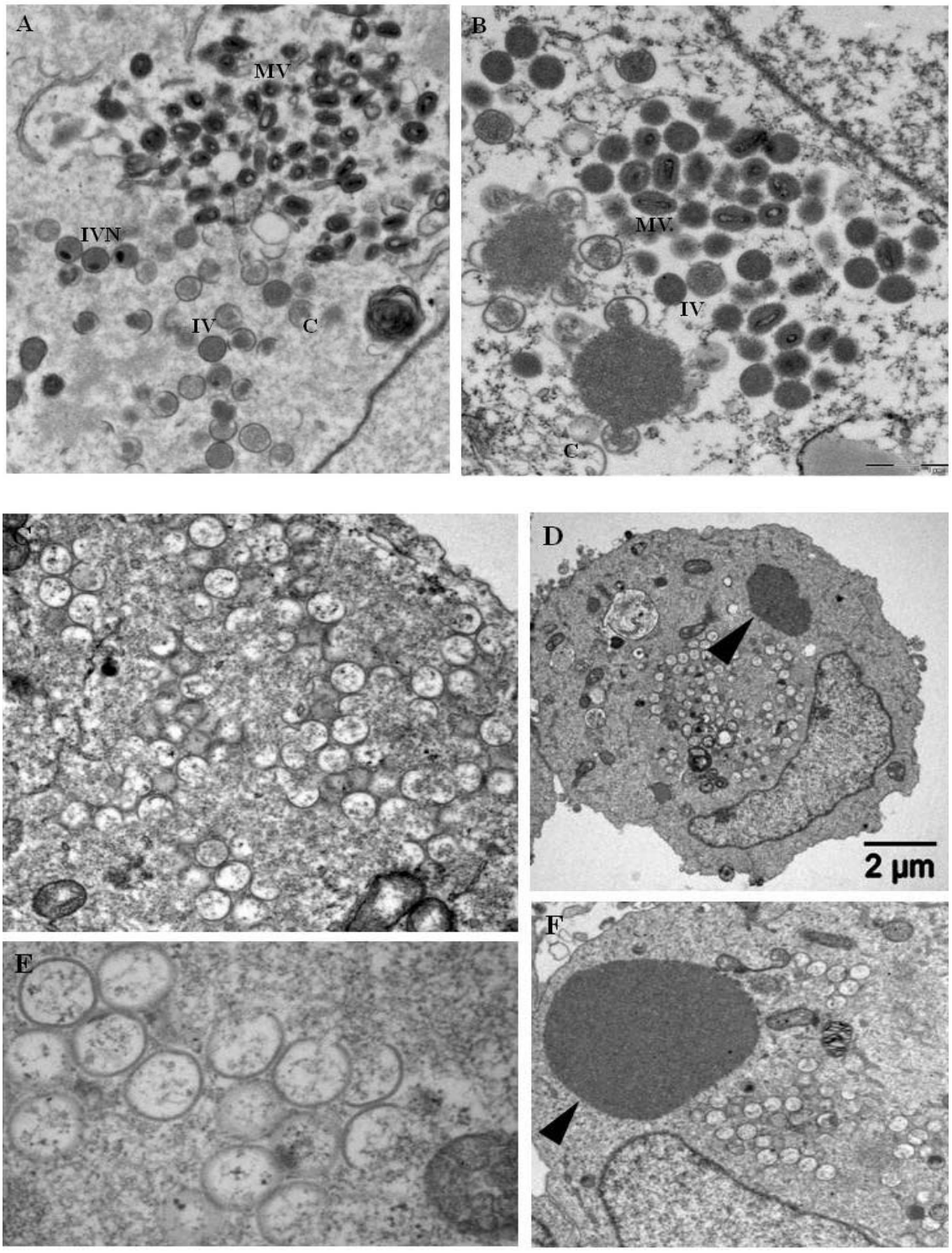

Figure 3-8. Electron microscopy of the vE6i mutant. A) and B) cells infected with vE6i in the presence of 50 µM IPTG, 24 hr. Infected cells are indistinguishable from wt virus infection. C-F) vE6i-IPTG infected cells, 24 hr. Empty IVs and "crystalloids" are evident. The arrowheads in D and F point to crystalloids.

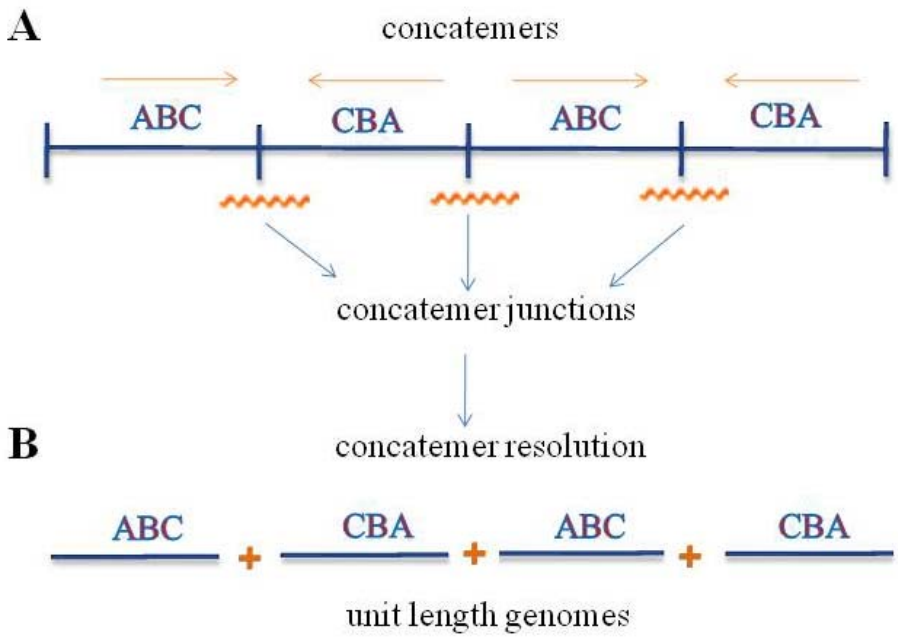

Figure 3-9. Scheme for DNA replication. A) In the vaccinia virus infection DNA replicates in a head-to-head, tail-to-tail fashion resulting in a large multi-genome molecule. B) The multi-genome molecule resolves into single genomes, catalyzed by a viral resolvase enzyme late during infection.

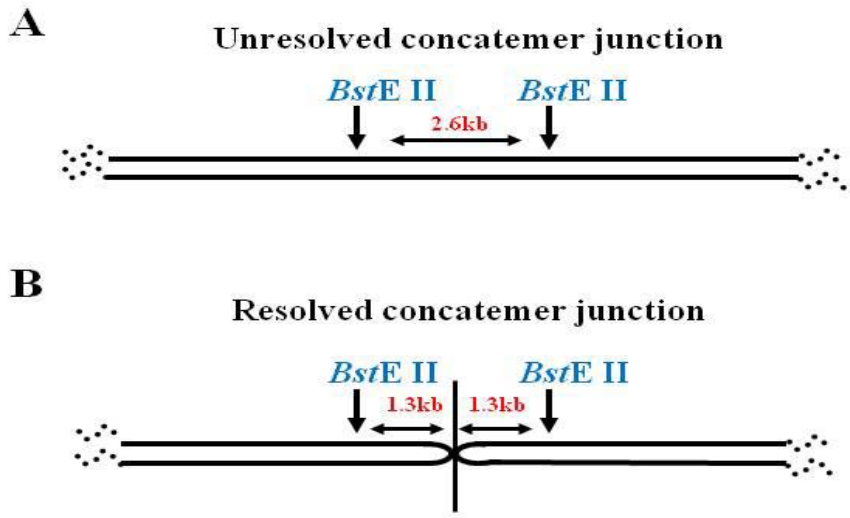

Figure 3-10. Vaccinia concatemer junctions. Digestion with *Bst*EII enzyme releases either A) 2.6 kb fragment is represent a concatemer junction, B) 1.3 kb fragments are signify mature hairpins.

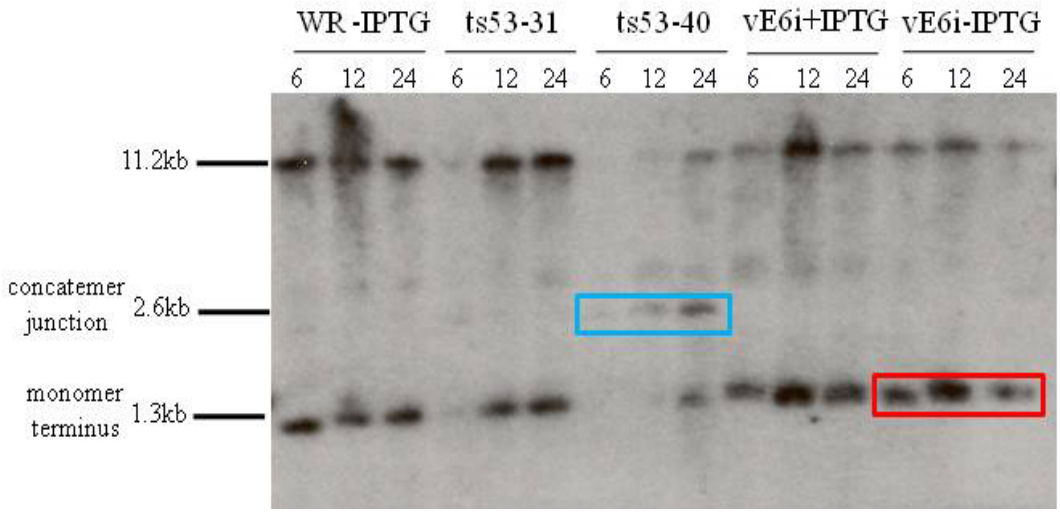

Figure 3-11. Concatemer resolution in infections with wild type WR, Cts53, vE6i viruses. BSC40 cells were infected with 10 pfu/cell in presence or absence of IPTG for vE6i, 31°C and 40°C for the wild type WR and the Cts53 mutant. Cells were harvested at designated times and hybridized with a [^{32}P] - labeled vaccinia DNA probe. The 1.3 kb and 2.6 kb bands are represent in resolved and not resolved concatemers respectively. The 11.2 kb band hybridized with the DNA probe was derived from repeat sequences in the vaccinia genome also present in the probe.

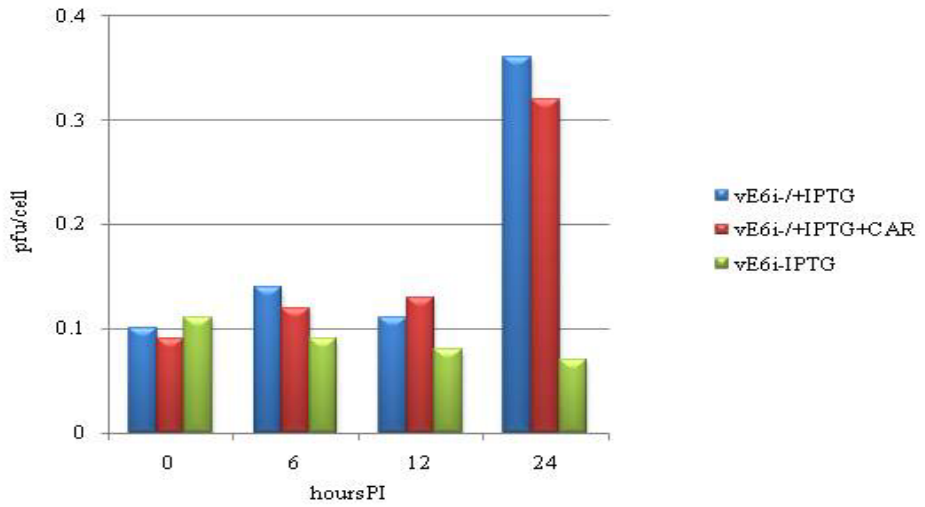

Figure 3-12. The vE6i block in assembly is reversible in absence *de novo* DNA synthesis. BSC40 cells were infected with 10 pfu/cell of vE6i in absence of IPTG for 24 hours, follow by addition of IPTG, or IPTG+CAR, or no IPTG. At various times, the cells were harvested and viral titers were measured by plaque assay. The X-and Y-axis indicate hours after infection at which cells were harvested and the infectious particle count, respectively.

CHAPTER 4
DISCUSSION

Poxviruses have left deep footprints in history as the causative agent for a disease with high mortality and as the base for vaccination, which began new era in medicine. The unique structure of poxviruses has attracted many scientists and led to extensive research in the past few decades. Substantial knowledge about poxviruses has been gained from the study of the vaccinia virus, which is used as a model to understand the biology of the poxvirus family. Approximately seventy gene products comprise the vaccinia virion; the functional impact of mutations in fifty genes which affect a virion morphogenesis has been characterized and the gene function revealed. The E6R gene product was identified among virion's structural proteins; however the E6R function is unknown. Understanding role of the E6R gene will advance our knowledge of poxvirus structure and assembly.

Two mutants Cts52 and vE6i were available to investigate the E6R gene function. Experimental data of the Cts52 phenotype was analyzed and the phenotype of the vE6i mutant was experimentally determined and examined in this study. Cts52 and vE6i displayed a normal phenotype in DNA and protein synthesis, that is, indistinguishable from wild type virus. However, whereas Cts52 showed normal protein processing and formation of MVs, vE6i revealed defective proteolysis and morphogenesis that arrested before IV formation. Particles purified from Cts52 infections done under non-permissive conditions were non-infectious and failed transcribe in vitro even though they packaged the normal complement of enzymes. In summary, the analysis of both mutants revealed that E6R is an essential gene expressed late during infection and plays a dual role in vaccinia morphogenesis: 1) transcription; 2) encapsidation of DNA into IVs.

Examination of the vE6i construct where the E6 protein is absent under a non-permissive condition showed that E6 is essential in the vaccinia life cycle. In the absence of an inducer, assembly of new infectious viral particles failed, suggesting an essential role of E6 in replication. This is consistent with the physical position of E6R in the vaccinia genome among the housekeeping genes, which are conserved throughout entire poxvirus family and historically have proven to be vital in virus life cycle. Also, it is consistent with alignments of every E6R gene in all poxviruses, which confirmed the E6R gene is conserved throughout entire poxvirus family and therefore essential.

To determine in what class in the temporal gene expression cascade the E6R gene belongs, the coding sequence of the E6 gene was examined. Both, the presence of a post-replicative gene transcription promoter signature sequence, TAAAA, just upstream of the E6R translation start codon, and the presence of T5NT early gene transcription termination sequences, suggests that E6R is a post-replicative gene. Post-replicative genes are defined as intermediate and late genes that are only transcribed after viral DNA replication. Furthermore, an antibody was raised against the E6 polypeptide and tested in the wild type and the Cts52 mutant infections by western blot. The kinetic analysis of E6 protein accumulation in viral infection showed that the E6R gene was expressed exclusively late during infection, thus assigning E6R to late temporal gene class (N. Moussatché, in preparation).

In the recent mass spectrometry of the purified virion particle, the E6 protein was identified (Chung et al., 2006; Yoder et al., 2006). To further investigate the E6 protein, vaccinia virions from wild type and Cts52 infection under permissive and non-permissive conditions were purified, treated with the neutral detergent NP40, and fractionated by centrifugation into a soluble fraction containing membrane proteins and insoluble fraction containing core proteins,

then tested with E6 antibody. The results showed that E6 is localized exclusively in a core fraction and is not found in the solubilized membrane fraction (N. Moussatché, in preparation). Based on an algorithm of the TransMem method (Sonnhammer et al., 1998), an alignment amino acid sequences with known transmembrane helices, predicted that the E6 protein has no transmembrane domains. The published experimental data demonstrate that most vaccinia membrane proteins possess transmembrane domains. Absence of transmembrane domain in E6 protein is consistent with finding of E6 in a core fraction of purified virion; therefore E6 is positively a core protein.

Transmission electron microscopy of the vE6i mutant under non-permissive conditions showed an arrested morphogenesis; only empty IVs were evident and dense crystalloids were observed in periphery of DNA factories. This phenotype correlates with the observation that the vE6i mutant is defective in the proteolysis of the precursor proteins p4a and p4b, which is essential for the formation of mature virions. By contrast, the Cts52 mutant under non-permissive condition was displayed a normal phenotype in proteolysis and assembly of mature virions. It seems that synthesis even nonfunctional E6 protein is enough to proceed to the next step in morphogenesis and the absence of E6 completely disrupts the assembly of new particles just before IV formation. A similar phenotype as the vE6i mutant under non-permissive conditions is observed with mutants in a "seven protein complex", containing the viral structural proteins F10, A30, G7, J1, D2, D3, A15(Szajner et al., 2001; Szajner et al., 2004a; Szajner et al., 2004b). Studies with seven protein complex mutants revealed a physical connection of these proteins with each other that forms complex essential for the association of viroplasm with IVs. Mutations in any gene in this complex resulted in the same phenotype as vE6i, specifically, the accumulation of empty IVs and formation of crystalloid aggregates. Also, a similar phenotype

described as DNA "paracrystals" and aberrant membranes was observed by Dales in temperature sensitive mutants in E6R gene, Dts41 and Dts80 that were characterized using electron microscopy (Dales et al., 1978), then later genetically analyzed and phenotypically characterized by Condit and co-workers (Lackner et al., 2003). As a result of phenotypic similarities of the seven protein complex and E6R gene, the role of E6R in the encapsidation of viroplasm into IVs is proposed. A search in the Poxvirus Bioinformatics Resource Center showed a computer-predicted DNA binding affinity of E6 protein that supports the hypothesis of the E6 plays role in physical association by binding of growing viral membranes with the viroplasmic DNA that results in formation of complete IVs. Similar to the E6 protein, the poxvirus database predicts a DNA binding affinity for the G7 protein. Also as E6 was found to be a core protein, previous studies with seven protein complex showed that all proteins except J1 found to be core proteins (Szajner et al., 2001; Szajner et al., 2004b). Even though E6 protein is apparently absent from the seven protein complex, the existence of another protein sub-complex that includes E6 is suggested. The absence of E6 might prevent protein-protein interaction within the sub-complex and disrupt its assembly that is essential to association of viroplasm and crescents to form immature virions. Therefore, E6 protein is crucial in the formation of MVs.

The fact that the absence of the E6 protein results in formation crystalloids and empty IVs suggested that the E6R gene may play a role in DNA resolution. In vaccinia virus, DNA replicates at early times during infection resulting in large multi-genome concatemers. The resolution of a multi-subunit DNA molecule into single genomes takes place late during infection and is a required event in order to package the genome and complete morphogenesis. The unresolved DNA is not able to package into IVs but instead is force to condense and form crystalloids that lead to arrested morphogenesis. The fact that the crystalloids contain DNA was

experimentally proven by anti-DNA labeling in infection with mutant in A10L gene (Heljasvaara et al., 2001). The A10L gene encodes precursor p4a core protein, proteolytic processing of which is critical to formation mature virions. The inducible mutant in A10L gene under non-permissive condition displayed a phenotype similar to vE6i, notably empty IVs and crystalloids. Even though the A10 mutant was normal in concatemer resolution, the replicated DNA was not able to package but instead condensed in large dense aggregates. The vE6i infection under non-permissive conditions was also normal for concatemer resolutions. Therefore, we conclude that formation of crystalloids is not a result of unresolved DNA concatemers.

To further understand the nature of crystalloids, we assessed whether the block to morphogenesis is reversible and whether the condensed DNA is viable for packaging in order to produce MVs. The infection was initiated under a non-permissive condition that normally results in blocked morphogenesis; later followed by incubation under permissive condition or under permissive condition in presence of a DNA replication inhibitor in order to assess the fate of existing DNA. In both cases, an equal amount of new infectious viral particles was formed indicating that blocked morphogenesis is reversible in presence of E6 protein. These findings suggest that mature virions can be formed from existing DNA, presumably from crystalloids. Although virus synthesis in a single round of wild type and vE6i infections at permissive conditions after 24 hours produced 38 pfu/cell and 50 pfu/cell respectively; in blocked morphogenesis after incubation for 24 hours at permissive conditions only 0.32 pfu/cell was produce. Considering this 100-fold difference, we assumed that in blocked morphogenesis some replicated DNA is scattered in viroplasm and not yet condensed into crystalloids could contribute to synthesis of new virus instead of DNA from crystalloids. The experimental data confirmed that in vE6i infection under non-permissive conditions, DNA replication was indistinguishable

from wild type. Therefore, the infection initiated even under non-permissive condition should contain enough DNA to form new virus. Therefore, if condensed DNA from crystalloids processed in order to make new virus, we should observed higher quantity of new virus synthesis. This finding suggests that crystalloids are possibly "terminal" even though the blocked morphogenesis is reversible to certain extent. To verify the source of DNA in formation of new virus, reversible morphogenesis should be repeated in parallel with electron microscopy to visualize if crystalloids are processed when permissive conditions are introduced. Also, construction of epitope-tagged E6 virus could serve as an effective tool to examine the E6 protein using electron microscopy and to determine if newly synthesized E6 protein is targeted to empty IVs or to crystalloids in order to trigger the condensed DNA to disaggregate and package to form MVs. The epitope-tagged E6 construct could also be used to immunopurify proteins associated with the E6 protein, which would revealed whether E6 is part of a protein complex involved in encapsidation of viroplasm. Also, the proteins of E6 complex could reveal more information about mechanism of viroplasm encapsidation.

We suggest that newly synthesized E6 protein functions as follows: a DNA binding sub-complex containing the E6 protein binds to replicated DNA forming a tubule-shaped nucleoprotein complex. Then the nucleoprotein containing the E6 sub-complex associates with the seven-protein complex, followed by genome encapsidation and formation of immature virions filled with viroplasm. The similar phenotype in mutants of seven-protein complex and the E6 protein suggests their possible association. To verify that E6 becomes the part of nucleoprotein complex, we could use the tagged E6 construct proposed above to localize E6 protein using electron microscopy.

In conclusion, based on experimental data, the E6R gene was shown to be essential in virus morphogenesis and play dual role as suggested by the vE6i mutant where E6 is essential in packaging the vaccinia genome into IVs; the Cts52 suggests that E6 is essential in transcription. The exact role of E6 protein is still unknown and further characterization of Cts52 and vE6i will provide more information about the E6R gene and extend our knowledge of poxviruses.

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BIOGRAPHICAL SKETCH

Olga Boyd graduated with BS and MS in mechanical engineering from Tomsk University, Russia. After moving to USA, she followed her life-long passion for biology. After completing her master's degree in molecular genetics and microbiology, she plans to pursue a career in biological sciences.