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The vaccinia virus E8R gene product is required for formation of transcriptionally active virions

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Abstract

Two vaccinia virus temperature sensitive mutants were mapped to the E8R gene and subjected to phenotypic characterization. Dts 23 contains a missense mutation in the coding region of E8R (L81F), and in Cts19 the initiating methionine codon is changed from ATG to ATA (M1I). The two ts mutants display normal patterns of gene expression and DNA replication during infection. The E8 protein is synthesized exclusively late during infection and packaged into virion cores Western blot analysis revealed that E8 synthesis is reduced in Dts23 infected cells at permissive (31 °C) and non-permissive temperature (39.7 °C) and absent in Cts19 infection under both conditions. Dts23 virions produced at 39.7 °C were indistinguishable in appearance from wt virions. Cts19 fails to produce identifiable viral structures when incubated at 39.7 °C. Purified Dts23 virions produced at 39.7 °C contain reduced amounts of E8 and have a high particle to infectivity ratio; purified Cts19 virions grown at 31 °C also show reduced infectivity and do not contain detectable E8. Dts23 grown at 39.7 °C could enter cells but failed to synthesize early mRNA or produce CPE. Soluble extracts from mutant virions were active in a promoter dependent in vitro transcription assay, however intact mutant cores were defective in transcription. We suggest that E8 plays a subtle role in virion core structure that impacts directly or indirectly on core transcription.

Keywords

Vaccinia virus; Poxvirus; Temperature-sensitive mutant; Virus genetics; Virus assembly; Virus Transcription

Introduction

The family *Poxviridae* comprises large dsDNA viruses that replicate in the cytoplasm of infected cells. Vaccinia virus (VACV), the prototype of this family, encodes more than 200 genes that function in viral RNA synthesis, genome replication, virion assembly and host defense (Moss, 2007). The vaccinia virion is complex and unusual in structure. The DNA genome is packaged in a biconcave, walled core structure which is flanked by protein-containing “lateral bodies”; the core and lateral bodies are encased in a proteolipid membrane

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to form a brick-shaped virion. The virion contains approximately 75 proteins, of which one third are enzymes involved in early mRNA synthesis, one quarter are membrane proteins, and the remainder are non-membrane structural proteins (reviewed in Condit et al., 2006). VACV enters cells either by fusion of the viral membrane directly with the host cell plasma membrane, or by endocytosis followed by fusion of the viral membrane with the vesicle membrane, in either case resulting in the release of the viral core and lateral bodies into the cytoplasm (Moss, 2006). The viral transcription machinery is then activated and early transcripts are synthesized and translated on cellular ribosomes. Early viral proteins catalyze DNA replication and intermediate viral mRNA synthesis; intermediate gene products in turn activate late gene transcription (reviewed in Broyles, 2003; Condit and Niles, 2002; Moss, 1994). The replicative cycle culminates with morphogenesis of new virions, assembled from late viral gene products (Condit et al., 2006).

As assessed by electron microscopy, the first indication of virus replication is the development of DNA enriched structures, named DNA factories, in the cytoplasm of infected cells (Condit et al., 2006). These factories have been shown to be the site of viral genome replication. Early during development, DNA factories are frequently surrounded membrane cisternae derived from the endoplasmic reticulum (ER) (Doglio et al., 2002; Tolonen et al., 2001). At later times when virion morphogenesis begins within the factories, the membranes surrounding the factories disappear. Virion morphogenesis within DNA factories begins with formation of viral membranes in the shape of crescents. Crescents evolve into spherical immature viral particles (IV), which ultimately morph into the brick shaped mature virions (MV) described above (Condit et al., 2006).

We report here the phenotypic characterization of temperature sensitive mutants in a vaccinia virion structural protein, E8. Our attention was drawn to E8 because of reports from Locker and coworkers (Doglio et al., 2002; Schramm and Krijnse-Locker, 2005; Tolonen et al., 2001; Welsch et al., 2003) suggesting that E8 might mediate the interaction between ER cisternae and developing DNA factories. These authors reported that E8 is expressed early during infection, contains two putative transmembrane domains, and is associated with membrane structures during infection, in particular with the ER cisternae surrounding early DNA factories. They reported further that E8 is a phosphoprotein whose phosphorylation is modulated during infection, and that it localizes to cores in MV. To further investigate the function of E8 in viral replication, we characterized two temperature sensitive (ts) mutants, Dts23 and Cts19, from the same complementation group (Lackner et al., 2003). Our phenotypic analysis of these mutants is generally inconsistent with a role for E8 early in VACV morphogenesis. We find instead that E8R mutants were able to form morphologically normal virus particles under non-permissive conditions; however purified mutant virions are significantly reduced in both infectivity and transcriptional activity. The possible role of the E8R gene product in VACV biology will be discussed.

Results

Mutations in the E8R gene confers the ts phenotype in Dts23, Dts25 and Cts19

The analysis of the merged Condit and Dales collections of VACV ts mutants has placed three mutants, Dts23, Dts25 and Cts19 in the same complementation group (Lackner et al., 2003). To map the mutations in these viruses to a single gene, we applied a one step marker rescue protocol using a library of PCR fragments of approximately 18 kbp that extend over the entire VACV genome (Yao and Evans, 2003). Briefly, confluent monolayers of BSC40 cells were infected with Dts23, Dts25 or Cts19 and transfected with the PCR products. Cells were incubated at 39.7 °C for 4 days, followed by crystal violet staining. The appearance of viral plaques in the monolayer indicates the rescue of the mutation. The results showed that all three mutations rescued with the PCR product YE3 that extends from gene F14L (WR53) to O1l

(WR68) (Fig 1 and data not shown). A second set of amplicons of approximately 5 kbp that cover the region defined by YE3 was used to refine the mapping. The results show that the lesion maps within the region defined by the PCR product LM13, comprising genes E6R (WR062) to E9L (WR065) (Fig 1 and data not shown). To finally map the mutations to a single gene, a third set of PCR products were prepared representing each individual gene from the region defined by LM13. Our results show that Dts23 (Fig. 1A) and Dts25 (data not shown) were rescued with the E8R amplicons.

Several attempts were made to rescue Cts19 with a single gene but all failed to provide a positive result. Therefore, a modified approach was taken in which adjacent pairs of open reading frames in a single amplicon for used for marker rescue. As shown in Fig. 1B the amplicon that includes both the E7R and E8R genes was able to rescue the Cts19 mutation.

To identify the precise mutation responsible for the ts phenotype in Dts23, Dts25 and Cts19, the complete E8R gene of each mutant was sequenced. In addition, the E7R gene was sequenced from the Cts19 mutant. Dts23 and Dts25 mutants have a C to T transition in nucleotide 241, substituting a phenylalanine for leucine at codon 81 (L81F). Thus both Dts23 and Dts25 have the same point mutation in the E8R gene and therefore the characterization of Dts25 was discontinued. The E7R gene from Cts19 was wt in sequence. In gene E8R, the Cts19 mutant has a G to A transition in nucleotide 3 substituting an isoleucine for the initiating methionine (M1I). The proximity of the Cts19 mutation to the 5’ end of the E8R gene is consistent with the fact that we were only able to rescue the mutant using an amplicon containing both the E7R and E8R genes. Furthermore, the Cts19 mutation abolishes the translation initiation codon of the E8R gene; the next possible initiation codon is 90 nucleotides downstream. Thus, we speculate that Cts19 is a null mutant.

One-step growth curve of Dts23 and Cts19

To analyze the growth characteristics of the mutants we performed one-step growth experiments. Since Dts23 mutant is in a VACV-IHDW background and Cts19 is in a VACV-WR background, we compared each mutant growth to its own parental type. BSC40 cells were infected with an MOI of 10 with either wild type or mutant virus at permissive and non-permissive temperatures. At different times post-infection, the cells were harvested and the amount of virus in each sample determined by plaque assay at 31 °C (Fig. 2). VACV-IHDW was able to grow at both 31 °C and 39.7 °C and produced a maximum of 63 and 66 pfu per cell after 48 or 36 hours post-infection, respectively. On the other hand, Dts23 produced 65 pfu per cell at permissive temperature but only 0.35 infectious particles per cell at non-permissive temperature. Cells infected with VACV-WR were also able to grow at permissive and non-permissive temperatures, and produced a maximum of 264 and 188 pfu per cell after 48 and 36 hours, respectively. Cts19 failed to generate a large amount of infectious particles at either temperature. At the permissive temperature, Cts19 produced a maximum of 4 pfu per cell, and only 1 infectious particle per cell was recovered at the non-permissive temperature. Since Cts19 replication was defective even at the permissive temperature, most of the remaining characterization of E8R gene function was done using Dts23.

DNA replication and protein synthesis are not affected in the ts mutants at the non-permissive temperature

To determine whether the mutation in the E8R gene would alter the synthesis of macromolecules, we measured the DNA accumulation and protein synthesis in cells infected at permissive and non-permissive temperatures. Cells were infected with the wt and Dts23 viruses at 31 or 39.7 °C and at various times after infection the cells were harvested and prepared for DNA slot blot analysis. Cells lysates were applied to a slot-blot membrane, hybridized to a ^{32}P-labeled vaccinia DNA probe, and quantified using a phosphor-imager (Fig. 3). The results

show that wt and mutant virus were able to synthesize DNA at the same level at both temperatures. It has been reported previously that Cts19 is not defective in DNA replication under non-permissive conditions (Condit and Motyczka, 1981).

Since DNA synthesis was not impaired in Dts23 incubated at 39.7 °C we next examined if there was any alteration in protein synthesis in cells infected with the mutant viruses. Cells were infected with wt and mutant virus at permissive and non-permissive temperatures and at various times after infection protein synthesis was evaluated by metabolic labeling with ^{35}S-methionine in a 30 minute pulse. Labeled proteins were separated by SDS-PAGE and analyzed by autoradiography (Fig. 4). We observed the typical pattern of protein synthesis in wt infected cells incubated at 31 and 39.7 °C. At 3 hours post-infection early viral proteins began to be synthesized and at the same time cellular protein synthesis began to shut-off. By six hours post-infection a new set of viral proteins representing post-replicative gene products appeared and persisted throughout the course of the infection. Dts23 infected cells incubated at both temperatures presented a pattern of protein synthesis indistinguishable from that which was observed in wt infected cells. Early viral proteins were detected at 3 hours post-infection and a late pattern was recognized at 6 hours post-infection and persisted throughout the course of infection. It has been reported previously that no modification of the pattern of viral protein synthesis is observed in Cts19 infected cells incubated at permissive and non-permissive temperatures (Condit and Motyczka, 1981).

Electron microscopic analysis of Dts23 and Cts19 infected cells

Transmission electron microscopy (TEM) has been used to identify the different steps of virus morphogenesis. The VACV maturation cycle is a relatively non-synchronous event and at late times of infection all stages of morphogenesis can be identified. To determine whether viral assembly was affected by the mutation in the E8R gene, BSC40 cells were infected with VACV mutants at the permissive and non-permissive temperature and at various times the cells were collected and processed for TEM (Fig. 5). No differences in virus formation were observed in cells infected with Dts23 at either the permissive or the non-permissive temperature (Fig 5A–D). All stages of virus morphogenesis could be observed Dts23 infected cells incubated at 31 °C (Fig 5A). In Dts23 infected cells incubated at the non-permissive temperature we could identify the presence of crescents, IV, IVN and MV with no perceptible difference in structure (Fig 5B–D). The presence of WV and EV could also be recognized in Dts23 infected cells at 39.7 °C (Fig 5 inset).

Similar to Dts23, no difference in virus morphogenesis could be observed in Cts19 infected cells and incubated at 31 °C where IV and MV were clearly identified (Fig 5E, F). However, when Cts19 infected cells were incubated at 39.7 °C we observed DNA factories and occasional aggregates of presumed aberrant virus structures (Fig. 5G), however we observed no MV formation and only rarely could IV structures were seen in the cytoplasm of cells after meticulous search (Fig 5G, H).

Kinetics of synthesis and accumulation of E8 in wt and mutant virus infected cells

It has been reported previously that the E8 protein is synthesized early during vaccinia infection (Doglio et al., 2002). However, inspection of the DNA sequence of the E8R gene reveals the presence of two early transcription termination sequences embedded well within the coding sequence, and a sequence consistent with an intermediate or late gene promoter immediately upstream of the coding sequence. We therefore thought it important to reinvestigate the kinetics of E8 synthesis during virus infection. BSC40 cells were infected with the wt and mutant viruses, incubated at permissive and non-permissive temperatures, and at various times after infection cells were harvested and the samples analyzed by western blot using an anti E8 anti-peptide antibody. Fig. 6 shows that in cells infected with either wt VACV or Dts23 at both

permissive and non-permissive temperatures, E8 protein appears at 6 hours post-infection, and persists throughout the course of the infection. The level of E8 accumulation in the Dts23 virus infections is significantly reduced compared to wt infection. No difference in E8 accumulation was observed in Dts23 infected cells incubated at permissive compared to non-permissive temperatures. As expected, in Cts19 infected cell there was no indication of E8 synthesis at any time during infection. The Western blot presented in Fig. 6 also shows a 35 kDa cross-reacting protein that accumulates late during infection. This protein was not present in mock-infected cells but was present in Cts 19-infected cells. We could not establish if this cross-reactive polypeptide was derived from a virus encoded gene or if it had a cellular origin that is induced during virus replication, however its presence in Cts19 infections clearly indicates that it is not derived from the E8 gene. Since a blast search of the 14- amino acids used to raise the E8 antibody did not reveal any other cellular or viral proteins besides the E8 ortholog, we assume that this signal was due to a non-specific reaction.

The kinetics of accumulation of E8 (Fig. 6) suggests that the E8R gene is expressed specifically late during infection. To verify the temporal class of E8R gene expression, cells were infected in the absence or in the presence of cytosine arabinoside (CAR) and at various times cells were harvested and processed for western blot analysis as described in Methods. Early protein synthesis is not inhibited by CAR. By contrast, intermediate and late gene expression is dependent DNA replication and therefore inhibited by CAR. Two VACV proteins were used as markers for early (gene F11L) or late (gene L4R) gene expression. In Fig. 7 we show that F11 appeared by 2 hours post-infection and its synthesis was not inhibited by the addition of CAR. One the other hand, L4 did not accumulate until 5 hours post-infection and its synthesis was inhibited by the drug. E8 displayed a late pattern of accumulation similar to L4, since the protein did not accumulate until the 5 hours post-infection and was inhibited by the addition of CAR. We conclude that E8R is a post-replicative gene, contrary to published reports (Doglio et al., 2002). The late expression of the E8 R gene has also been confirmed by R. Jeremy Nichols and P. Traktman (personal communication).

Characteristics of Dts23 and Cts19 the purified particles

To examine further the mutant virus particles we purified virions from infected cells incubated permissive and non-permissive conditions. Virions were purified by sucrose gradient sedimentation from infected-cells incubated at 31 and 39.7 °C and from Cts19 infected-cells incubated at 31 °C, as described in Methods. As shown in Table 1 wt and mutant viruses produce approximately the same number of particles per cell regardless of the temperature of incubation. However the specific infectivity (particles/pfu) of the isolated particles can vary depending on the mutant or the incubation conditions. The specific infectivity of Dts23 grown at 39.7 °C is reduced nearly 35 fold compared to the specific infectivity of the same virus produced at 31 °C. The specific infectivity of Cts19 particles grown at 31 °C was 38 fold lower than wt virus grown under the same conditions. No significant difference in specific infectivity was observed between wt grown at both permissive and non-permissive temperatures, and Dts23 grown under permissive conditions had a specific infectivity equivalent to wt virus.

The protein content of purified virions was examined by SDS-PAGE (Fig. 8). Comparing the WR and IHDW strains, two differences in protein composition could be easily identified in the ~ 32 kDa molecular weight ranges. However no conspicuous difference between wt and mutant virions could be observed within each strain using this analysis. The content of DNA in purified virions was examined by pulse-field electrophoresis. No differences in size and in the content of DNA per particles were observed comparing wt and mutants grown at different conditions (data not shown).

Doglio et al (2002) reported that E8 partitioned into the core fraction after treatment of virions with detergent and a thiol reducing agent. To confirm this observation and to analyze the

integrity of the mutant particles, virions were treated with NP-40 and DTT, core/precipitate and membrane/soluble fractions were separated by centrifugation and the samples were analyzed by western blot (Fig. 9). Our results show that indeed E8 partitioned in the core fraction and there was no difference in the amount of E8 present in wt virions compared to Dts23 virions purified from 31°C infections. A lower amount of E8 was observed in Dts23 virions purified from 39.7°C infections and E8 was absent from Cts19 virions. The analysis of other viral proteins present in core and membrane fractions revealed no difference in quantities when wt and mutant virus were compared. A27, a viral membrane protein, partitioned into the supernatant and two core proteins, L4, a single stranded DNA and RNA binding protein, and H4, an early-stage transcription factor partitioned into the core fraction.

Mutant particles can penetrate BSC40 cell but do not induce cytopathic effect and do not synthesize early viral mRNA

To determine whether Dts23 virions purified from 39.7°C infections could initiate a cycle of infection we examined whether the virions were able to penetrate cells, induce a cytopathic effect and synthesize early viral mRNA. To analyze the entry process BSC40 cells were infected with virus for 30 minutes at 4 °C. After this adsorption period, the inoculum was removed, medium containing 100 μg/ml of cycloheximide was added to the cells and incubation was continued at for 2 hours at 31 °C. The presence of viral cores inside the cells was detected by confocal microscopy after labeling the cells with anti-A4 antibody. As shown in Fig. 10D and F, Dts23 virions purified from 39.7°C infections were able to enter cells with an efficiency equivalent to control infections (Fig 10A–C). These results show that the mutation in the E8 protein does not affect binding of virions to cells, or uptake of virions into cells and uncoating.

We next examined the ability of Dts23 virions purified from 39.7°C infections to induce CPE in infected cells. For this purpose cells were infected with wt or mutant virions purified from cells infected under permissive or not permissive conditions. Infected cells were then incubated under permissive conditions and the CPE was evaluated 18 hours post-infection. Dts23 virions purified from 39.7°C infections were not able to induce CPE while the other samples produce extensive alteration in the cell morphology (data not shown).

Finally, we analyzed whether Dts23 virions purified from 39.7°C infections have the ability to direct synthesis of early viral transcripts *in vivo*. Cells were infected with wt or mutant virions purified from cells infected under permissive or not permissive conditions. Infected cells were then incubated under permissive conditions, total RNA was isolated at 3, 6 and 9 hours post-infection and the presence of early mRNA was analyzed by northern blot (Fig. 11A). As expected, early transcription was detected in wt infected cells at both 31°C and 39.7°C, and in Dts23 infected cells at 31°C. By contrast, no early mRNA was detected up to 9 hours following infection with Dts23 virions purified from 39.7°C. We conclude that Dts23 virions purified from 39.7°C infections are defective in early viral mRNA synthesis *in vivo*.

Mutant particles are defective in core mRNA synthesis

Vaccinia virions package all the enzymes necessary for synthesis, capping and polyadenylation, of early viral mRNA, and extrusion of the mRNA from the virion core (Moss, 1994). Transcriptional activation can be achieved *in vitro* by incubating virions with a non-ionic detergent, a reducing agent and nucleoside triphosphates (Kates and McAuslan, 1967). Extruded RNA can be separated from the core by centrifugation, where the core associated RNA is recovered in the pellet and the released RNA is partitioned in the supernatant (Kato et al., 2004b). To evaluate the transcription capacity of the mutant viruses, virions purified from cells infected under permissive and non-permissive conditions were activated and assayed for RNA synthesis (Fig. 11B). VACV IHDW virions grown at both the permissive and non-

permissive temperature synthesized viral RNA at the same rate, and equivalent amounts of RNA were synthesized after 60 minutes of incubation (Fig. 11B, left). Although the total amount of RNA synthesized by the WR strain was lower than the IHDW strain, the kinetics of RNA synthesis in both cases were comparable (Fig 11B, right). Dts23 virions purified from 31°C infections were able to synthesize RNA at the rate similar to WT virions grown under the same conditions and similar amounts of transcripts accumulated. However Dts23 virions purified from 39.7°C infections were defective in transcription and produced only 10% the amount of RNA produced by wt IHDW virions (Fig 11B, center). Cts19 virions were also defective in transcription, producing only 25% the amount of RNA produced by wt WR virions (Fig.11B, right). The small amount of RNA synthesized by Dts23 and Cts19 mutant virions was extruded from cores normally (data not shown).

Soluble virion extracts from mutant particles can transcribe DNA in a template dependent system

To determine whether Dts23 virions purified from 39.7°C infections contain the normal complement of viral RNA polymerase and transcription initiation factors, a sodium deoxycholate soluble virion extract was prepared and assayed for promoter-specific transcription. Virion extracts were incubated in the presence of ^{32}P-rNTP with a linearized dsDNA containing a vaccinia-specific early promoter upstream of a 375 nt G-less cassette. As shown in Fig. 11C extracts from both wt and mutant virions purified from cells infected under both permissive and non-permissive conditions were able to synthesize the expected 375 nt RNA transcript. We conclude from this experiment that the mutant virions contain the normal complement of active viral RNA polymerase and early transcription initiation factors.

Discussion

To better understand the role of E8 in the VACV replicative cycle we have characterized two temperature sensitive mutants in this gene. The two mutants are radically different in genotype, and have related but distinct phenotypes. Dts23 produces reduced amounts of the E8 protein at both permissive and non-permissive temperatures; it packages significantly reduced amounts of E8 protein into virions at the non-permissive temperature and normal amounts at the permissive temperature. It is not clear at this point whether the transcriptional defect found in Dts23 virions produced at the non-permissive temperature results from the reduced amount of E8 present in virions, or from the fact that the E8 protein packaged is mutant, or both. In Cts19 the translation initiation codon for the E8 protein is changed from the wt ATG to ATA, and it produces no detectable E8 protein at either the permissive or the non-permissive temperature; Cts19 therefore is formally a null mutant. Consistent with this genotype, the virus grows very poorly even at the permissive temperature and not at all at the non-permissive temperature. Interestingly, the phenotype of Cts19 at the permissive temperature resembles the phenotype of Dts23 at the non-permissive temperature: at 31°C Cts19 produces a normal burst of virions, however the virions produced have a low infectivity and the virions are defective in transcription in vitro. The results from these two mutants together suggest that when the E8 protein is limiting in amount or absent or poorly packaged, then depending on the temperature of incubation, transcriptionally defective virions are produced which are otherwise morphologically normal in appearance. At the non-permissive temperature, Cts19 presents yet another phenotype. Gene expression and DNA replication are unaffected, and viral DNA factories are formed, however virus assembly seems to be interrupted at a very early stage, with only occasional immature particles and aberrant structures present. This suggests that in addition to a role in virion transcription, the E8 protein may also play a role early during virus assembly. The question of a dual role for E8 during infection might ultimately be addressed by use of a mutant carrying an inducible copy of the E8 gene. Based on our results we would predict that such a mutant would have a temperature sensitive phenotype when E8 expression

is repressed. Use of an inducible mutant to titrate the amount of E8 present during infection might provide further insight into possible roles of E8 in morphogenesis and in virion transcription.

Locker and coworkers first observed that vaccinia DNA factories are temporarily wrapped in ER membrane early during their evolution and they suggested that this process is mediated at least in part by the E8 protein (Doglio et al., 2002; Schramm and Krijnse-Locker, 2005; Tolonen et al., 2001; Welsch et al., 2003). E8 was first implicated in ER wrapping of viral DNA factories through a screen of the vaccinia genome for proteins which contained transmembrane domains and which, based on promoter sequence analysis, might be expressed early during infection. E8 does contain two putative transmembrane domains, and subsequent studies by the same group suggested that the protein was indeed expressed early, and that it decorated the ER membranes that surround viral DNA factories (Doglio et al., 2002; Schramm and Krijnse-Locker, 2005). Locker and co-workers also reported that E8 is a phosphoprotein and a target for the viral F10 kinase in vitro, and that it has DNA binding activity (Doglio et al., 2002). It should be noted that the evidence that E8 is actually embedded in membranes in infected cells is inconclusive. While immunofluorescence and immunoelectron microscopy localize E8 to the nuclear envelope, ER, and ER surrounding the DNA factories, E8 is not efficiently inserted into membranes either co-translationally or post-translationally, and it does not behave like an integral membrane protein as assessed by sodium carbonate extraction of membranes from infected cells. It therefore seems possible that the two hydrophobic regions in E8 in fact do not function as transmembrane domains, but rather that these regions give the protein hydrophobic character relevant to its localization and function in the virus core.

Our results are largely inconsistent with a role for E8 in mediating ER wrapping of viral DNA factories. First, our results clearly show that E8 is expressed late during infection, and reexamination of the E8 DNA sequence is consistent with this finding, revealing both internal early gene transcription termination signals and upstream late promoter elements. Second, if ER wrapping of viral DNA factories were an obligatory feature of viral replication, one might expect that a mutant defective in this process would be defective in viral DNA replication or formation of DNA factories. In fact, at the non-permissive temperature Cts19 mutants are interrupted at an early stage of morphogenesis, a phenotype which is not completely inconsistent with a role for E8 in evolution of DNA factories. However, at 31°C Cts19 forms mature, transcriptionally inactive virions in the apparent absence of E8 protein, and Dts23 displays a similar phenotype at 39.7°C. Thus the bulk of our phenotypic analysis points away from a role for E8 in DNA factory structure or function and rather toward a role in the fine structure and/or transcriptional activity of mature vaccinia virions, discussed below.

Vaccinia early gene transcription has been studied in vitro in two significantly different experimental contexts: using intact virion cores or using a soluble transcription system. As described in Fig. 12, virion cores can be activated by treatment of virions with a neutral detergent and a reducing agent such that they become permeable to added nucleoside triphosphates (Kates and McAuslan, 1967). Activated cores synthesize, cap and polyadenylate authentic early viral mRNA and extrude the RNA from the cores in an energy dependent fashion (Moss, 1994). The soluble system (Fig 13) consists of a deoxycholate extract of cores (Moussatche, 1985; Rohrmann and Moss, 1985). This enzyme extract is capable of accurate transcription initiation and termination from an exogenously added DNA template, and the extract also contains the enzymes required for capping and polyadenylation of this RNA. All of the activities required for mRNA synthesis and modification in the soluble system have been identified, including initiation factors, termination factors, the RNA polymerase, the capping enzyme, and the poly (A) polymerase (reviewed in Broyles, 2003; Condit and Niles, 2002; Moss, 1994). Importantly, E8 has not been identified as a component of the soluble transcription system, and our results show that extracts from our E8 deficient virions are

competent for promoter specific transcription in vitro. Interestingly, several VACV gene products have been identified which are not required for transcription or RNA modification in the soluble system but are required for transcription in activated cores. These proteins fall into two categories. One category includes proteins which are clearly structural in nature and have an overt impact on virion maturation. Mutations in such proteins cause production of aberrant virions that are transcriptionally inactive but contain all the enzymes required for accurate transcription in a soluble system (Kato et al., 2004b). In another category, proteins have been identified which when mutated produce virions that are indistinguishable in appearance from wt particles and contain the normal complement of transcription enzymes as assayed in the soluble system, but the virions produced, while capable of entering cells, are transcriptionally inactive and hence non-infectious. The mutants which produce virions with this latter phenotype affect two classes of core proteins, specifically proteins with an enzymatic activity or proteins that have exclusively a structural role. Proteins in the enzymatic class include the dual phosphatase (H1L) (Liu et al., 1995), topoisomerase (H6R) (da Fonseca and Moss, 2003) or the nucleoside triphosphate phosphohydrolase II (I8R) (Gross and Shuman, 1996); mutants in all of these proteins can form particles at non-permissive condition that are indistinguishable from wt but are deficient in early transcription. These enzymes therefore probably provide functions essential for transcription in the unique environment of the core particle, but are not required for transcription in the soluble system. Proteins in the structural class include L3L or L4R genes (Resch and Moss, 2005; Wilcock and Smith, 1996); mutants in both these proteins were able to form virions at non-permissive condition that are unable to synthesize RNA. Although RNA synthesis in mutant virions produced by these viruses was reduced *in vivo* and in the core transcription system, both mutants contained an active transcriptional apparatus that recognized an early promoter based on assays of soluble transcription extracts. However, a significant difference was observed between L4R and L3L mutants. Despite the fact that RNA synthesis in both mutants was reduced from 4 to 20 times respectively, in the L4R mutant there was also impairment in the release of the transcripts from the core; this phenomenon did not occur in the L3L mutants. E8 mutants resemble L3L mutants since in these mutants core RNA synthesis was drastically reduced but the transcripts synthesized were released from the cores.

Structural considerations may provide additional clues to the function of the E8 protein. E8 has two hydrophobic domains, it is a basic protein that can bind to DNA, and it can be phosphorylated in vitro by the viral F10 protein kinase. Proteins with these characteristics have been implicated directly or indirectly in transcription in different cell systems (Berger, 2002; Leo and Chen, 2000; Nowak and Corces, 2004). Phosphorylation of histone H3 has a role in transcription and in chromosome condensation (Nowak and Corces, 2000; Nowak and Corces, 2004). In Drosophila and in cultured mouse cells, the induction of the heat-shock gene is associated with a significant increase in histone H3 phosphorylation and an alteration on the transcription rate (Dyson et al., 2005b; Dyson et al., 2005a). Nuclear receptor phosphorylation is also associated with a modification of transcription and an alanine to serine substitution results in a reduction of transcription activity (Leo and Chen, 2000; Wu et al., 2005). Thus, E8 has characteristics consistent with a DNA binding protein that could modulate virion core transcription.

The vaccinia core contains additional DNA binding proteins whose function in virus replication is only partially understood, notably F17R (VP11) and L4R (VP8) (Kao et al., 1981; Kao and Bauer, 1987; Nowakowski et al., 1978a; Nowakowski et al., 1978b; Yang et al., 1988; Yang and Bauer, 1988). F17R is a major DNA-binding protein and has preferential binding to supercoiled DNA but can also bind to ssDNA and dsDNA. F17R is phosphorylated on serine residues and its phosphorylation is minimally dependent on the viral F10 kinase and modulated by the viral H1 phosphatase and probably by cellular kinases (reviewed in Condit et al., 2006). Mutants in the F17R gene grown under non-permissive conditions fail to produce MV

but instead forms abnormal IV with aberrant internal structures that accumulates in the cytoplasm of infected cells (Zhang and Moss, 1991). L4 binds to ssDNA, dsDNA and cooperatively to RNA, and as described above essential for formation of transcriptionally competent viral particles (Bayliss and Smith, 1997; Wilcock and Smith, 1994). L4R mutants under non-permissive conditions produce abnormal IV but maturation can also proceed to produce MV that are indistinguishable in appearance from wt virions but are 100 fold reduced in infectivity.

Evidence suggests that transcriptional competence of the vaccinia virion core is tightly coupled to core structure. Mutants in structural genes A3L, L3L, L4R and now E8R all form particles that contain an RNA polymerase complex that is active in a soluble transcription system but inactive in a core transcription system Mutants in the major structural protein A3 (p4b) form morphologically aberrant particles that have clearly matured beyond the IV stage but lack properly formed cores. Mutants in the L4 protein, described above, form normal looking MV however these particles show decreased stability in the presence of deoxycholate, suggesting a subtle defect in virion core structure. Mutants in the L3 and now E8 proteins form normal looking particles that like the A3 and L4 mutants, are unable to synthesized RNA (Kato et al., 2004b; Resch and Moss, 2005; Wilcock and Smith, 1996). Based on these comparisons, we speculate that the function of E8 protein in virion core, and probably L3 and L4, is to play a subtle role in virion core structure that impacts directly or indirectly on core transcription. Ultimately, elucidation of the precise role of these proteins in vaccinia infection will require a highly refined description of vaccinia virion core structure and function.

Materials and Methods

Cell and virus culture, plaque assay, one-step growth

BSC40 cells, a continuous line of African green monkey kidney cells, were used throughout this work. Wild-type vaccinia virus strains WR and IHDW were used as a control for the characterization of the temperature mutants: Cts-19, Dts-23 and Dts-25 (Condit et al., 1983; Condit and Motyczka, 1981; Lackner et al., 2003). The conditions for cell and virus culture, virus infection, plaque titration, and one-step growth have been described elsewhere(Condit et al., 1983; Condit and Motyczka, 1981). The non-permissive temperature for mutant infections was maintained at 39.7 °C; the non-permissive temperature is labeled as 40 °C in some figures for convenience.

Viral DNA and plasmid

For sequencing of the E8R gene total infected cell DNA was isolated using Qiagen DNeasy miniprep spin columns (Qiagen) and followed the manufacturer's instructions for isolation of DNA from cells in culture. Plasmid pSB24, which contains a synthetic early vaccinia promoter upstream from a 375 nucleotide G-less cassette, was obtained from Dr. Stewart Shuman (Memorial Sloan Kettering Institute). pSB24 was linearized by digestion with S*ma*I (NEBiolabs), purified (Roche - PCR purification kit) and analyzed in a 0.8% agarose-TAE gel.

DNA sequence analysis

PCR amplification products from total DNA preparations, as described above, were used to sequence the wild-type and mutants viruses. Primers that hybridize outside of the coding sequence were constructed to amplify a 1647-bp product. Sequence was acquired using amplification primers and primers that annealed within the coding region. Sequencing was performed by the University of Florida ICBR DNA Sequencing Core Laboratory.

Marker rescue

One-step marker rescue was accomplished as described before with some modifications (Thompson and Condit, 1986). Briefly, 60-mm dishes of BSC40 cells were infected with each ts virus at an appropriate moi determined empirically by terminal dilution for 1 hour at 39.7 ° C. After this time, the inoculum was removed and 4 ml of OPTI-MEM was added to the culture. The cells were transfected with 1.5 μg of a PCR product using Lipofectamine reagent (Invitrogen) and incubated for 4 days at non-permissive temperature. After this time, the dishes were stained with crystal violet. For the initial mapping experiments, PCR amplifications were performed using a library of primers designed to create 13 overlapping 20 kbp products that collectively span the vaccinia genome (Yao and Evans, 2003), as shown on the top of Fig. 1. Once a region on the vaccinia genome was identified a second set of PCR products of 5 kbp each were used based on the library described by (Luttge and Moyer, 2005). Once the mutated region was identified individual open reading frames in that 5 kbp region were amplified from genomic viral DNA and used in a subsequent marker rescue experiment to map each mutant to an individual gene.

Analyses of VV DNA slot-blot hybridization

Confluent 35-mm dishes of BSC-40 were infected with VACV as described. At different times postinfection cells were harvested and extracts were prepared as described previously (Damaso et al., 2002). Samples of each extract were applied in triplicate to a Hybond-N membrane (GE-Healthcare) using a Slot Blot Minifold II apparatus (Schleicher & Schuell, Inc.). After filtration, the DNA was denatured *in situ* and probe with a ^{32}P end-labeled oligonucleotide mixture that represents several genes from the VAC genome. Hybridization carried out for 18 h at 42°C in hybridization solution 67(6 × SSC; 0.1% SDS; 10× Denhardts. 100 μ/ml ssDNA. The blots were washed threw times in 5 × SSC, 1% SDS, once at 30°C, and twice at 50°C and analyzed with a phosphor-imager apparatus (Storm 860, GE-Healthcare).

Metabolic labeling of viral proteins

Metabolic labeling of proteins was performed by incubating infected cells with ^{35}S-labeled amino acids mixture (ProMix – GE Healthcare) in a methionine free media for 30 minutes. The cells were resuspended in SDS-sample buffer (50 mM Tris-HCl, pH 6.8; 1% SDS; 10% glycerol; 140 mM 2-mercaptoethanol; 0.1% bromophenol blue) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and autoradiography was done as described previously (Condit and Motyczka, 1981; Kato et al., 2004b).

Purification and analysis of virus particles

Six 150-mm dishes containing confluent monolayers of BSC40 cells were infected with either VACV-IHD-W or Dts-23 viruses at a moi of 10 at the permissive or the non-permissive temperature. Cells incubated at 39.7 °C were harvested 24 h after infection and the cell incubated at 31 °C were harvested after 72 hours of infection. Cell-associated virus was purified by differential centrifugation through a 36% sucrose cushion followed by banding on a 24–40% sucrose gradient as previously described (Ausubel et al., 1994; Kato et al., 2004b). Similar procedure was done with VAC-WR and Cts-19 but the viruses were incubated only at 31°C.

Western blot analysis

Virion core and envelope extracts were prepared and analyzed by incubating 0.06 A_{260nm} units (0.7 μg) of wt or mutant particles with core buffer (100 mM DTT and 1 % NP40 in 50 mM Tris–HCl pH 8.0) for 30 minutes at 37 °C. Core and membrane fractions were partitioned by a 15 minutes centrifugation at 20.000 × g. The fractions were resuspended in SDS-sample buffer and the proteins were separated by a 12 % SDS-PAGE and transferred to a nitrocellulose membrane as described (Black et al., 1998). The membranes were blocked with a solution of

TBS-T (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 0.01% Tween 20) and 5% non-fat dry milk (TBS-TM) for 1 hour, washed twice with TBS-T and incubated with an appropriate dilution of the primary antibody in TBS-TM for 2 hours. Peptide base rabbit polyclonal antibodies specific for the E8 protein (amino acids 177 to 190) were prepared by Sigma Genosys Inc, and used at a dilution of 1:1000. The dilution and source of the other antibodies was the following: anti-F11 (1:1000) (Kato et al., 2004a); anti-L4 (1:40.000) rabbit serum was supplied by Dr. Dennis Hruby (Oregon State University); anti-H4 (1:5000) rabbit serum was supplied by Dr. Bernard Moss (NIAID); anti A-27 (1:40.000) monoclonal antibody supplied by Dr. David Ulaeto, Department of Biological Science, DSTL, Porton Down, Salisbury, UK).

Electron microscopy

BSC40 cells on 60-mm dishes were infected with either wt or mutant viruses at a moi of 10 at either 31 or 39.7°C. Samples from 24 to 48 post-infection were prepared for transmission electron microscopy with assistance of the University of Florida ICBR Electron Microscopy Core Laboratory (Kato et al., 2004b).

Confocal microscopy

BSC40 cells grown on an 8-well glass slide (BD-Falcon) were infected with 1,000 particles per cell for 1 hour at 4°C. After this incubation the unabsorbed virus was removed, the cells were washed once with PBS and incubated at 31°C in DMEM containing 100 μg/ml of cycloheximide for 2 hours. Next the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, and incubated for 1 hour with anti-A4 antibody (Dr Mariano Esteban., Centro Nacional de Biotecnologia, CSIC, Madrid, Spain). Cy2 labeled goat-anti-rabbit antibody (Jackson Immunoresearch Laboratories, Inc.) was used as secondary antibody for visualization of A4 and DNA staining was stained with DAPI (Vectashield, Vector Laboratories, Inc, CA). The slides were observed on a Leica Confocal microscope (Leica TCS SP5).

RNA isolation and northern blotting

BSC40 cells were infected with wt and mutant virus at a moi of 1000 particles per cell. At indicated times points the cells were washed with PBS and total RNA was isolated using an RNeasy kit (Qiagen) following the protocol described by the manufacturer. For the northern blot procedure, RNAs were mixed with RNA sample loading buffer (Sigma-Aldrich), denatured by incubating at 70 °C for 10 minutes, and loaded onto a 1.2% agarose gel containing 2.2 M formaldehyde and 1 × MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). Samples were electrophoresed at 80 V for 90 minutes. The gel was soaked in water, treated with 0.05 N NaOH, and twice washed in 20 × SSC (1 × SSC = 15 mM sodium citrate, 150 mM sodium chloride) for 20 min at each step. The RNAs were transferred over night to a GeneScreen neutral charge membrane (PerkinElmer, Boston, MA) and processed as described (Cresawn et al., 2007).

Transcription by permeabilized virions

Permeabilized virion transcription reactions were done essentially as described previously (Kato et al., 2004b). 0.4 A_{260nm} units of virus were incubated in a reaction mixture containing 60 mM Tris-HCl pH 8.0, 0.05% Nonidet P40 (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 (100 μCi/μmol), at 31°C. At various times 50-μl aliquots were removed, precipitated directly with 5% TCA, filtered onto a glass microfibre filters (934-AH, Whatman), and the radioactivity was determined by liquid scintillation counting.

Preparation and assay of soluble virion transcription extracts

Soluble transcription extracts were prepared and assayed as described by Moussatché (1985) with modifications. All extraction and chromatography steps were carried out at 4 °C. 5.0 A_{260nm} units of virus was resuspended in 800 µl of core buffer (50 mM Tris–HCl pH 8.0, 10 mM DTT, 0.05% NP40) and incubated for 10 minutes at room temperature. The cores were collected by a 2 min centrifugation in an Eppendorf microfuge, and resuspended in 150 µl of Buffer N (300 mM Tris-HCl, pH 8.0, 250 mM KCl, 50 mM DTT). Deoxycholate was added to a final concentration of 0.15% and the mixture incubated for 30 minutes on ice with occasionally gentle mixing. Glycerol was added to a 10% final concentration and the extract was centrifuged for 5 minutes at 20,000 × g. The supernatant was collected and applied to a 150 µl DEAE column equilibrated in Buffer M (50 mM Tris-HCl, pH 8.0, 250 mM KCl, 1mM EDTA pH 8.0, 0.1% Triton X-100,3 mM DTT). 100 µl fractions were collected and assayed for non-specific RNA-polymerase activity in 100 µl reactions containing: 60 mM Tris–HCl, pH 8.0, 10 mM DTT, 3 mM $MnCl_2$, 1 mM ATP, 1 mM GTP, 1 mM UTP, 0.1 mM α ^{32}P-CTP (25 µCi/µmol), 1 µg ssDNA (from bacteriophage M13) and 8 µl of enzyme. The reactions were incubated for 30 minutes at 31 °C and the radiolabelled RNA was measured by precipitation with 5% TCA. Peak fractions were pooled and stored in aliquots at −80 °C. Promoter-specific transcription by virion extracts was assayed as follows: 100 µl reactions containing 40 mM Hepes-KOH, pH 8.0, 5 mM DTT, 6 mM $MgCl_2$, 2 mM spermidine, 2 mM ATP, 2 mM UTP, 0.01 mM α ^{32}P-CTP (3000 Ci/mM), 0.24 µg pSB24 DNA linearized with Sma I and 2 to 6 µl of enzyme were incubate at 31 °C for 15 minutes. Reactions contained equivalent amounts of RNA polymerase activity, determined by performing a time course of nonspecific transcription with varying amounts of virion extract. RNA was purified from transcription reactions by proteinase K treatment and phenol/chloroform extraction and ethanol precipitation, resuspended in RNA SB (95% formamide, 20 mM EDTA, 0.1 µg Bromophenol blue, 0.1 µg of xylene cyanole) and analyzed by electrophoresis on 6 % denaturing polyacrylamide/urea gels.

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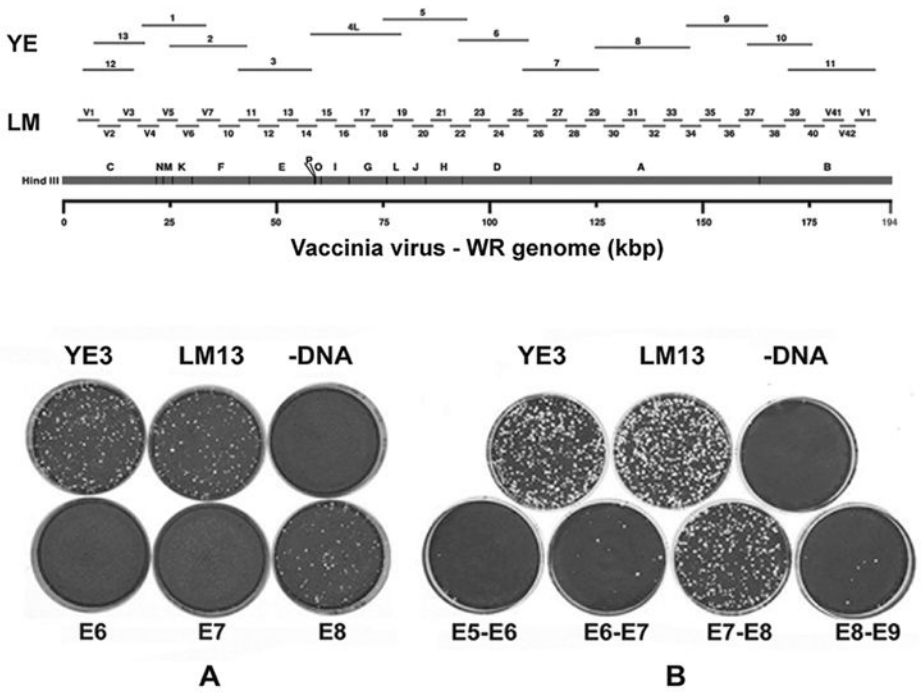

Fig. 1. One-step marker rescue of E8R mutants

BSC40 cells were infected with Dts23 or Cts19 mutant virus and transfected with a PCR product as described in Methods. Crystal violet-stained dishes are shown, labeled with the PCR amplification used in the transfection as shown in the drawing on the top of the Figure. (A): Dts23; (B): Cts19.

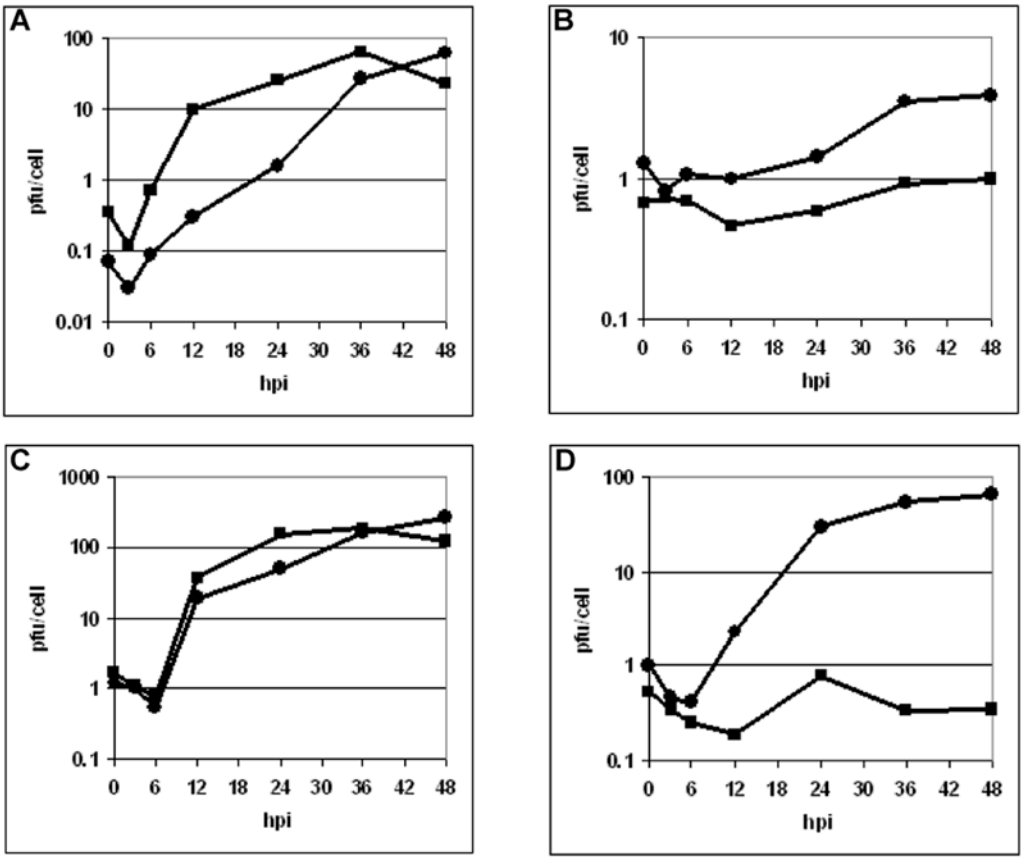

Fig. 2. One-step growth of wt, Dts23 and Cts19 viruses

BSC40 cells were infected at moi = 10 pfu/cell with wt or mutant viruses, incubated at 31 or 39.7 °C for the indicated periods of time (abscissa), and virus yields were determined by plaque titration at 31°C (ordinate). (A) WR-wt; (B) Cts19; (C) IHDW-wt; (D) Dts23. (31°C - ●; 39.7° C - ■)

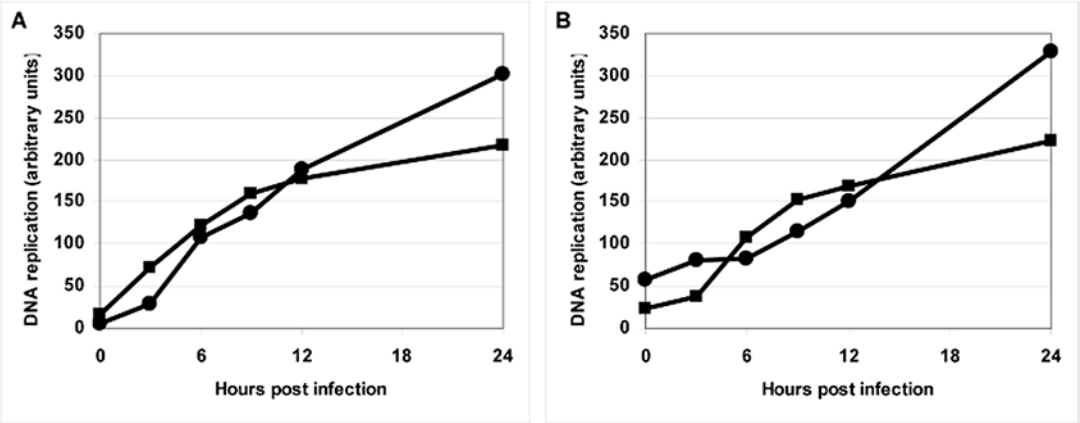

Fig. 3. Viral DNA replication in virus infected cells

BSC-40 cells were infected at moi=10 pfu/cell, incubated at 31 or 39.7 °C, and at indicated times postinfection, cell extracts were prepared and applied on nylon membranes, and hybridization to a ^{32}P labeled vaccinia virus DNA probe was performed as described under Methods. The numbers (arbitrary units) express the average value of three different measurements. (A) IHDW-wt; (B) Dts23. (31°C - ●; 39.7°C - ■)

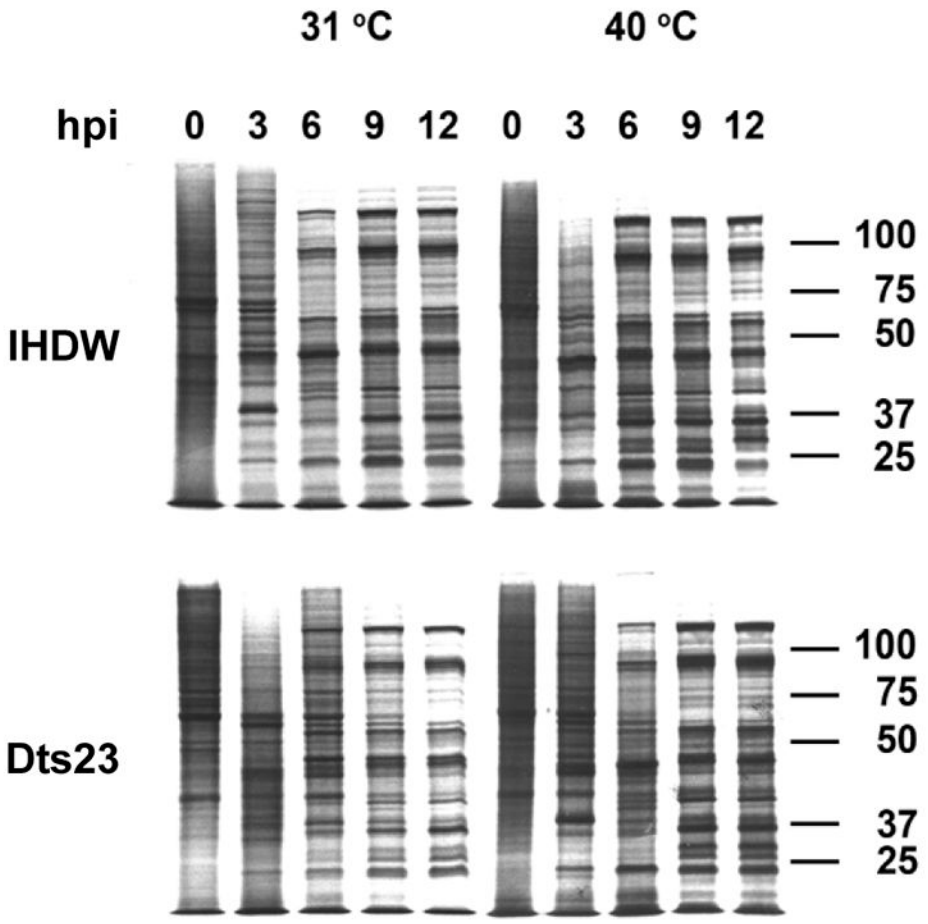

Fig. 4. Protein synthesis in wt and mutant-infected cells

BSC40 cells were infected at moi = 10 pfu/cell, incubated at 31 or 39.7 °C, and pulse-labeled with ^{35}S methionine at various times postinfection, indicated in hours at the top of each autoradiogram. Cells lysates were electrophoresed on SDS-PAGE, and gels were dried and autoradiographed. Incubation temperature and time of the pulse label is indicated at the top of each column, and the mutant used in the infection is indicated to the left of each row of autoradiograms. Approximate molecular weights, in kDa, are indicated to the right of each autoradiograms.

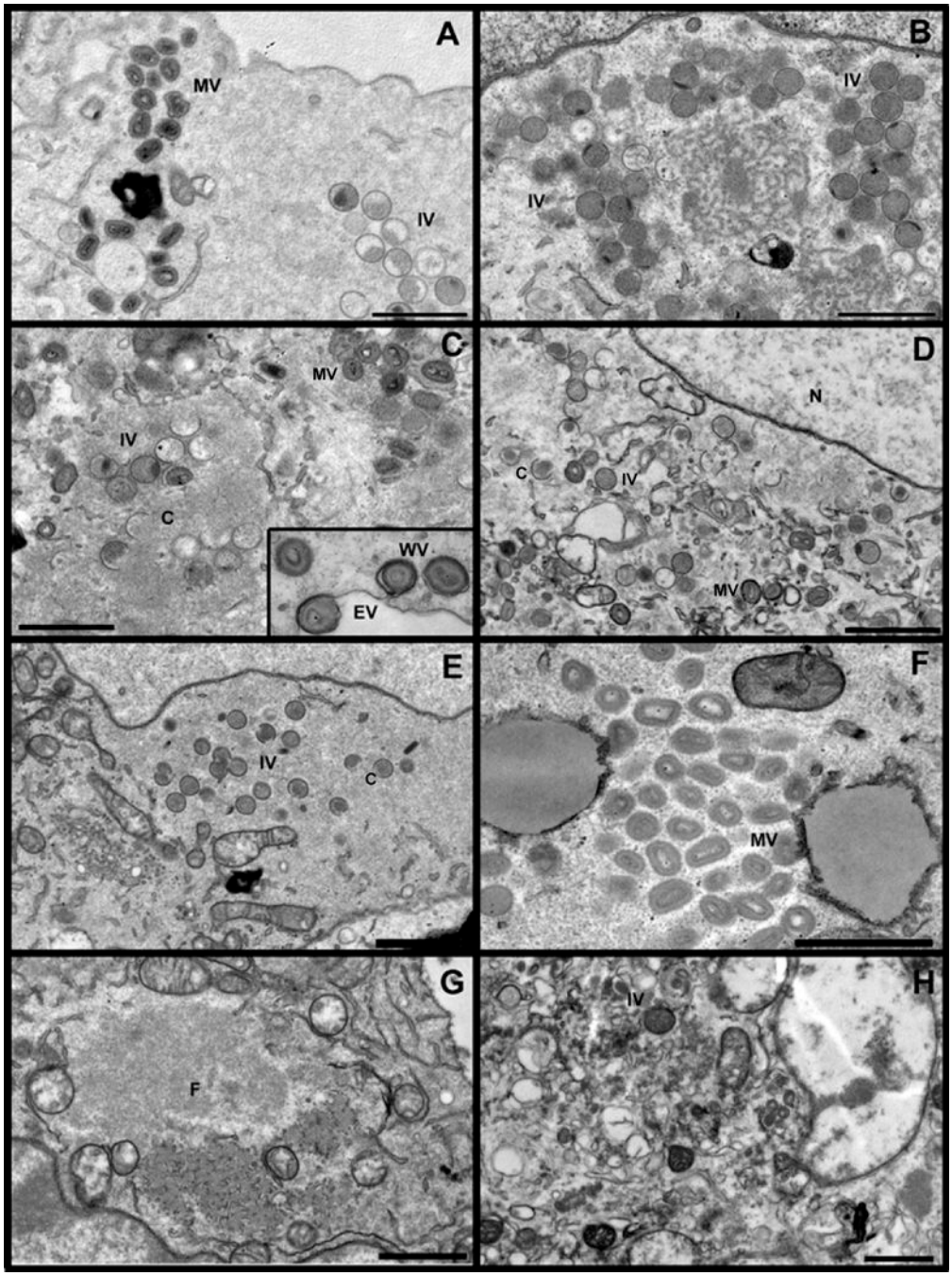

Fig. 5. Electron micrographs of wt and mutant-infected cells

BSC40 cells were infected with wt or mutant virus at a moi = 10 pfu/cell, incubated at 31 or 39.7 °C for 16 hs (D); 24 hs (A–C, E, G; inset); 36 hs (H) and 48 hs (F) and processed for electron microscopy as described in Methods. IV= immature virions; MV= mature virions; WV= wrapped virions; EV= extracellular virions; C= crescents; N= nucleus; F= DNA factories. (A) Dts23 infections at 31 °C; (B – D) Dts23 infections at 39.7 °C; (E – F) Cts19 infections at 31 °C; (G – H) Cts19 infections at 39.7 °C. The inset in C shows the formation of WV and EV in Dts23 infections. Bar= 1 μm

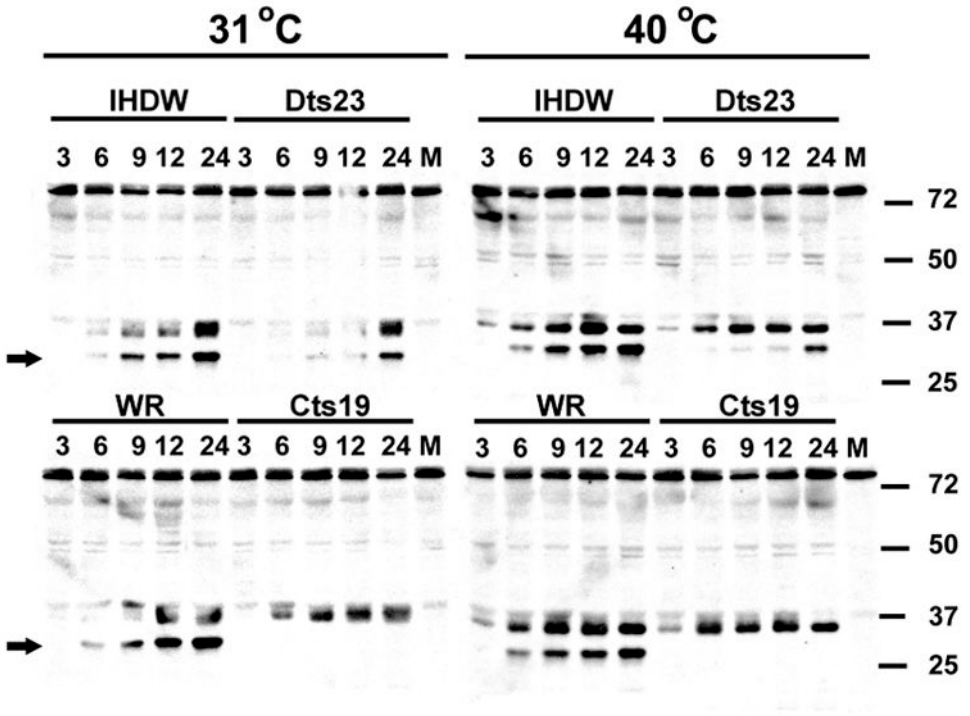

Fig. 6. Accumulation of E8 in wt and mutant virus infected cells

BSC40 cells were infected with wt or mutant virus at a moi = 10 pfu/cell and incubated at 31 or 39.7 °C. At different times, indicated in hours on the top of each lane, samples were removed and processed for Western blot as described under Methods. The arrow represents the expected MW for the E8 protein. Approximate molecular weights, in kDa, are indicated to the right of the F.

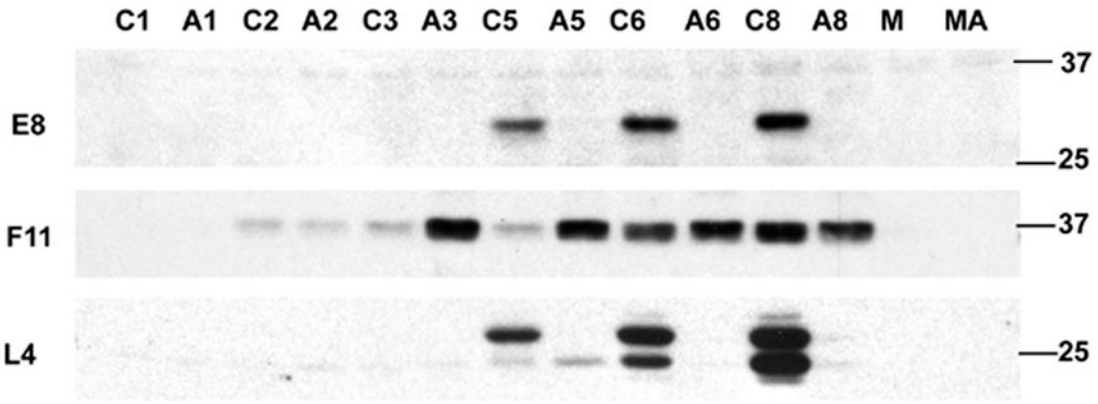

Fig. 7. Kinetics of synthesis of E8

BSC40 cells were infected with VACV-WR at a moi = 10 pfu/cell and incubated at 37 °C in the absence (C) or in the presence of 40 μg/ml of CAR (A). At 1, 2, 3, 5, 6, and 8 hours post-infection samples were removed and prepared for Western blot as described in Methods. M and MA are mock infected cells incubated in the absence or presence of CAR. Approximate molecular weights, in kDa, are indicated to the right of the autoradiograms.

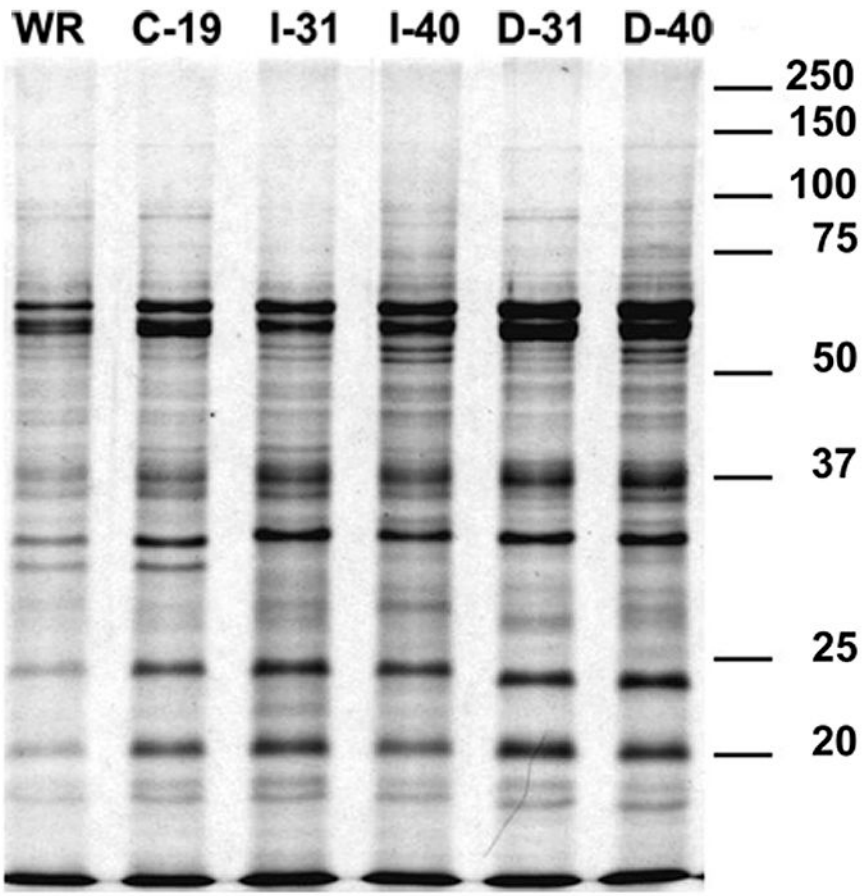

Fig. 8. Analysis of proteins of purified wt and mutants virions

0.18 A_{260nm} units (12 μg) of purified virions were resuspend in SDS-sample buffer, the proteins were separated in an 11% SDS-PAGE and stained with Coomassie blue as described in Methods. The virion analyzed in labeled on the top of each lane: WR, wt; C-19, Cts19 grown at 31°C; I-31, IHDW grown at 31 °C; I-40, IHDW grown at 39.7 °C; D-31, Dts23 grown at 31 °C; D-40, Dts23 grown at 39.7 °C. Molecular weights markers are indicated to the right of the gel, in kDa.

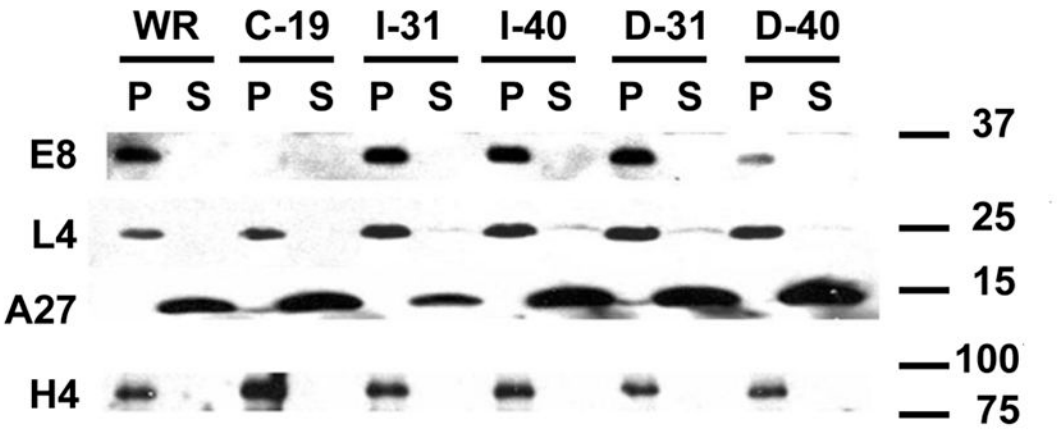

Fig. 9. Western blot analysis of virions core and membrane fractions

0.06 A_{260nm} units (0.7 μg) of each virion sample, labeled as described in Fig.8, was resuspended in core buffer and processed as described under Methods. The gene product corresponding to each antiserum used is indicated to the left of each row; the virus sample probed is indicated on the top of each lane where P= pellet/core fraction and S= soluble/membrane fraction; the molecular weight markers are indicated on the right side of the blot, in kDa.

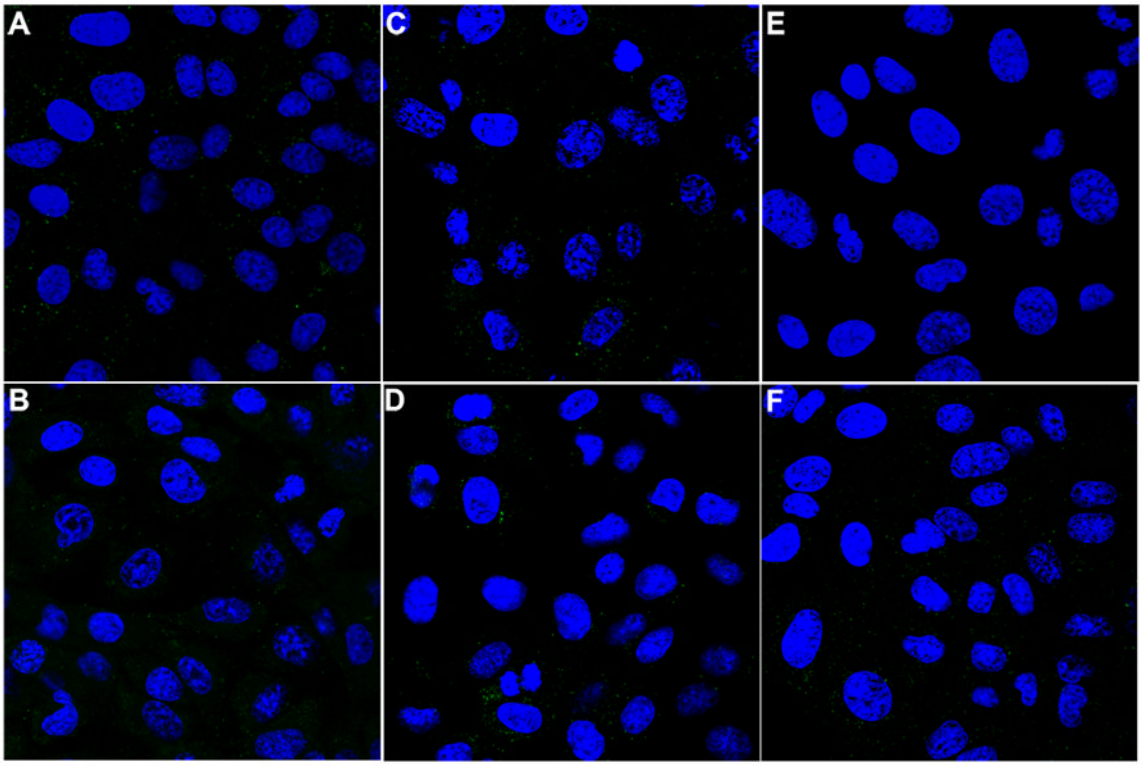

Fig. 10. Analysis of entry process of wt and mutants

BSC40 cells were infected with moi = 1000 particles of each virus grown at 31 °C (A, C) or 39.7 °C (B, D, F), fixed, permeabilized, stained for the viral core protein A4 (green) and for DNA(blue), and analyzed by confocal microscopy as described in Methods. IHDW= A, B; Dts23= C, D, F; mock infected (E).

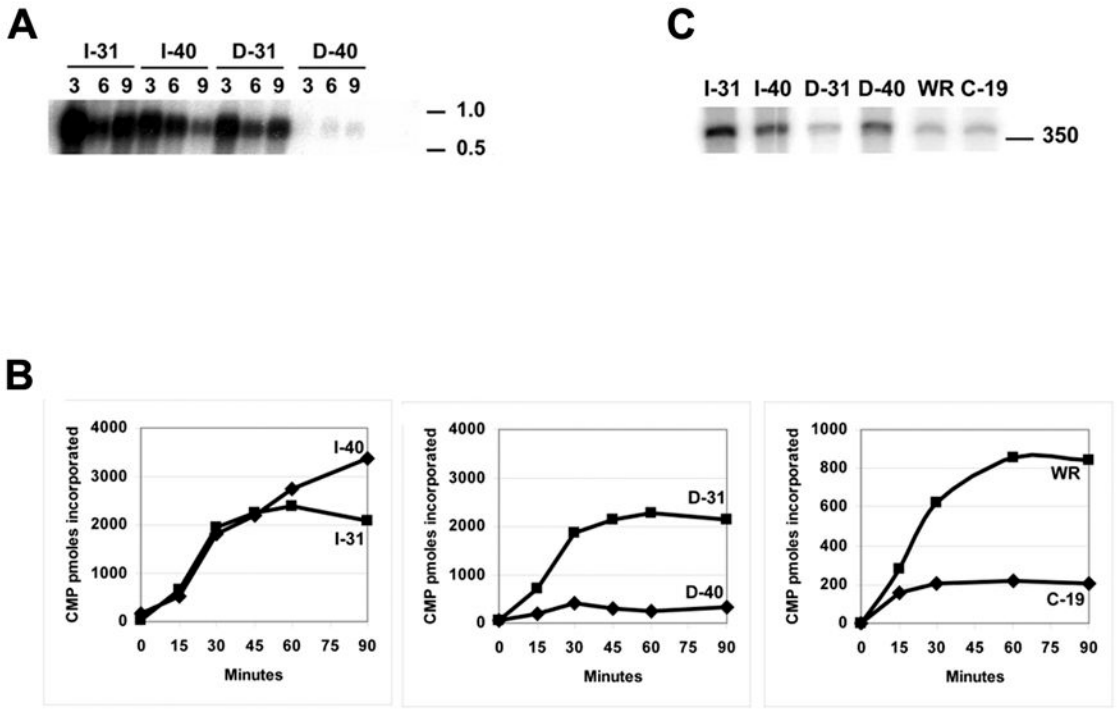

Fig. 11. In vivo and in vitro RNA synthesis of purified virions

A) Northern blot analysis of wt and mutants infected cells. BSC40 cells were infected with the indicated virus at moi = 1000 particles per cell and incubated at 31 °C. At 3, 6 and 9 post-infection samples were removed and prepared for Northern blot. Following pre-hybridization, 1×10^7 cpm of a riboprobe, synthesized from the C11R gene, was added to the blot and incubated overnight at 55 °C as described in Methods. The virion analyzed in labeled on the top of each lane: WR, wt; C-19, Cts19 grown at 31°C; I-31, IHDW grown at 31 °C; I-40, IHDW grown at 39.7 °C; D-31, Dts23 grown at 31 °C; D-40, Dts23 grown at 39.7 °C; the molecular weight markers in the right in kb.

B) RNA synthesis directed by purified wt and mutants particles. Purified virions were permeabilized with NP40 and DTT treatment, incubated with [α^{32}P] CTP and the other nucleotides for 90 minutes. At indicated times, samples were removed, and the acid precipitable radioactivity was determined as described in Methods. The virions were labeled as above.

C) Specific early transcription directed by wt and mutant virion extracts. Soluble transcription extracts were prepared by incubating purified VACV cores with sodium deoxycholate and passing through a DEAE-cellulose column. The active fractions were pooled, assayed for specific early transcription, and the product analyzed by electrophoresis on denaturing polyacrylamide gel as described in Methods. The virions were labeled as above; the molecular weight marker in indicated at the right in nucleotides.

Table 1

Characteristics of purified wt and mutant Vaccinia virus particles

Virus–Temperature[a]	Yield (Particles/cell)[b]	Infectivity (particles/pfu)
WR- 37 °C	3150	19
Cts19- 31°C	1000	737
Dts23-31 °C	1736	22
Dts23- 40 °C	1344	775
IHDW-31 °C	852	9
IHDW-40 °C	988	11

[a] WR wt virus grown at 37 °C from a low moi infection; Cts19 virus grown at 31°C from a low moi infection. IHDW and Dts23 were isolated from high moi infections. Viruses grown at 39.7 °C were incubated for 24 hours; viruses grown at 31 °C were incubated for 72 hours.

[b] Calculated from an A_{260nm} reading of purified virus, assuming a 1 A_{260nm} =1.2×10^{10} particles/ml.