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Virology 375 (2008) 236–252
VIROLOGY
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Phenotypic analysis of a temperature sensitive mutant in the large subunit of the vaccinia virus mRNA capping enzyme

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Received 5 December 2007; returned to author for revision 17 December 2007; accepted 21 January 2008
Available online 4 March 2008

Abstract

The heterodimeric vaccinia virus mRNA capping enzyme is a multifunctional enzyme, encoded by genes D1R and D12L. Published biochemical experiments demonstrate that, in addition to mRNA capping, the enzyme is involved in early viral gene transcription termination and intermediate viral gene transcription initiation. This paper presents the phenotypic characterization of Dts36, a temperature sensitive mutant in the large subunit of the mRNA capping enzyme (G705D), encoded by gene D1R. At the non-permissive temperature, Dts36 displays decreased steady state levels of some early RNAs, suggesting a defect in mRNA capping. Mutant infections also show decreased steady state levels of some early proteins, while DNA replication and post-replicative gene expression are absent. Under non-permissive conditions, the mutant directs synthesis of longer-than-normal early mRNAs from some genes, demonstrating that early gene transcription termination is defective. If mutant infections are initiated at the permissive temperature and shifted to the non-permissive temperature late during infection, steady state levels of intermediate gene transcripts decrease while the levels of late gene transcripts remain constant, consistent with a defect in intermediate gene transcription initiation. In addition to its previously described role in mRNA capping, the results presented in this study provide the first *in vivo* evidence that the vaccinia virus mRNA capping enzyme plays a role in early gene transcription termination and intermediate gene transcription.

Keywords: Vaccinia virus; mRNA capping; Temperature sensitive mutant; Transcription

Introduction

Vaccinia virus, the prototypical member of the orthopoxvirus family, is a double stranded DNA virus (192 kb) which encodes approximately 200 genes (Moss, 2001). Vaccinia virus replicates in the cytoplasm of infected cells and therefore encodes most of the factors required for viral DNA replication and viral transcription. Viral transcription is temporally regulated and genes are divided into early, intermediate, and late classes. Early genes are transcribed in the infectious particle, which contains all of the necessary factors for transcription in newly infected cells. Intermediate and late transcription both occur in cytoplasmic viral DNA factories and are coupled to viral DNA replication. Early transcripts encode factors that are required for DNA replication and intermediate gene transcription. Intermediate transcripts encode late transcription factors and late transcripts encode early transcription factors that are packaged in the new virions as well as factors needed for virion maturation and morphogenesis. Vaccinia virus possesses two forms of the viral RNA polymerase specific for early and postreplicative (intermediate plus late) gene transcription. The early and the postreplicative form of the polymerase share eight subunits in common; the early form of the polymerase has one additional subunit which is required along with early transcription factors for recognition of early promoters (Ahn and Moss, 1992; Zhang et al., 1994). Early genes possess a distinct termination signal in the nascent mRNA transcript (U_5NU); thus, early transcripts have defined 3′ ends (Yuen and Moss, 1987). Intermediate and late transcripts each have stage-specific promoters recognized by cognate trans acting factors. The mechanism of transcription termination for intermediate and late transcripts differs from the mechanism of early termination in that the early termination

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doi:10.1016/j.virol.2008.01.028

signal does not function as a termination signal in intermediate and late transcription. The lack of a well-defined termination signal in intermediate and late transcripts leads to 3′ end heterogeneity in these transcripts. All viral transcripts are capped and poly-adenylated regardless of the heterogeneity of their 3′ ends.

Vaccinia virus possesses a multifunctional, virally encoded mRNA capping enzyme. The capping enzyme is a heterodimer comprised of a large and a small subunit (Guo and Moss, 1990; Martin et al., 1975). The large subunit (97 kDa) is encoded by the gene D1R and the small subunit (33 kDa) is encoded by the gene D12L (Morgan et al., 1984; Niles et al., 1989). Biochemical analysis illustrates that the enzyme is responsible for the capping of nascent viral mRNA transcripts (Ensinger et al., 1975; Martin et al., 1975) and is a factor needed for both early transcription termination (Luo et al., 1991; Shuman et al., 1987; Shuman and Moss, 1988) and intermediate transcription initiation (Harris et al., 1993; Vos et al., 1991). The capping enzyme has also been implicated in telomere resolution by *in vivo* analysis of temperature sensitive mutants in both subunits of the capping enzyme (Carpenter and DeLange, 1991; Hassett et al., 1997).

The structure of the mRNA cap is a 7-methylguanosine (m^7G) linked through an inverted triphosphate bridge to the initiating nucleoside of the nascent mRNA transcript. The mRNA cap structure is formed by a series of four enzymatic reactions:

i. $\mathrm{pppN(pN)_n \rightarrow ppN(pN)_n + P_i}$ (RNA triphosphatase)

ii. $\mathrm{ppN(pN)_n + GTP \rightarrow GpppN(pN)_n + PP_i}$ (mRNA guanylyltransferase)

iii. $\mathrm{GppN(pN)_n + AdoMet \rightarrow {}^{7m}GpppN(pN)_n + AdoHcy}$ (mRNA (guanine − 7) methyltransferase)

iv. $\mathrm{{}^{7m}GpppN(pN)_n + AdoMet \rightarrow {}^{7m}GpppNpN_m(pN)_{n-1} + Ado\ Hcy}$ (mRNA (nucleoside − $O^{2'}$) methyltransferase)

In vaccinia virus, the first three reactions in cap formation are catalyzed by the viral capping enzyme and the fourth reaction is catalyzed by a different, virally encoded enzyme, the product of gene J3R. The active sites for the three enzymatic reactions catalyzed by the capping enzyme in vaccinia virus are contained in the large subunit of the capping enzyme. The active sites for the RNA triphosphatase and mRNA guanylyltransferase activities are both located in the amino-terminus (amino acids 1–545) of the large subunit (Guo and Moss, 1990; Higman

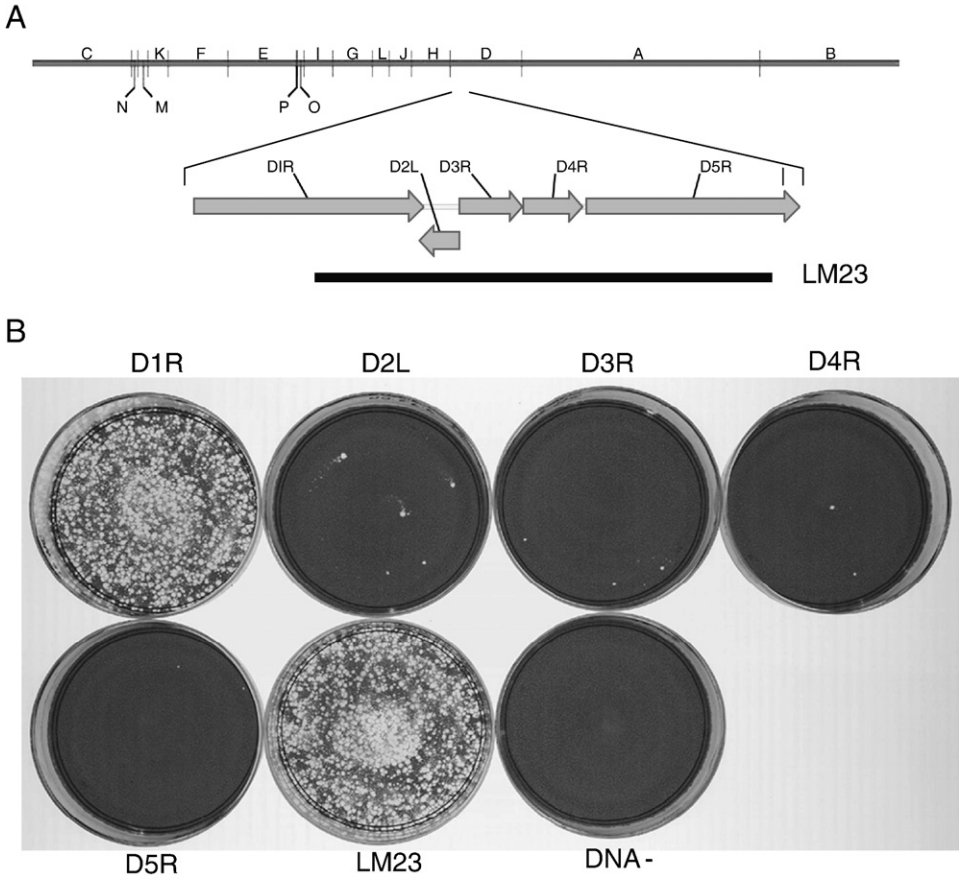

Fig. 1. Marker rescue of the Dts36 temperature sensitive virus. (A) A Hind III restriction map of the vaccinia virus genome. The blown up region below the map shows the genes that comprise LM23, the 5 kb PCR product that successfully rescued the Dts36 infection at 39.7 °C. (B) Crystal violet stained dishes are shown labeled with the PCR product that was used in the transfection.

et al., 1992; Shuman, 1990; Shuman and Morham, 1990) but have been shown to encompass distinct domains (Myette and Niles, 1996; Yu and Shuman, 1996). Association of the large subunit with the small subunit is not required for the RNA triphosphatase and mRNA guanylyltransferase enzymatic activities (Shuman, 1989; Shuman and Morham, 1990). The methyltransferase active site is located on the carboxyl-terminus (amino acids 540–844) of the large subunit of the capping enzyme (Higman et al., 1994; Mao and Shuman, 1994). The association of the D1 and D12 subunits of the capping enzyme is critical for the methyltransferase activity of the capping enzyme (Higman et al., 1992; Mao and Shuman, 1994). Both subunits of the vaccinia capping enzyme are also required for the early transcription termination function although none of the capping enzymatic activities are required for this function (Luo et al., 1995; Yu et al., 1997). The intermediate transcription initiation function of the capping enzyme also requires both subunits of the capping enzyme (Vos et al., 1991) and like the termination activity is independent of the guanylyltransferase activity (Harris et al., 1993).

In the current study, we describe the phenotypic characterization of two temperature sensitive vaccinia virus mutants which both have a mutation in the large subunit of the vaccinia virus capping enzyme and which comprise a new complementation group (Lackner et al., 2003). Further characterization of one of the two viruses, Dts36, shows that this virus possesses an early phenotype. In Dts36 at the non-permissive temperature, early mRNA metabolism is defective, early protein expression is irregular, and both DNA replication and post-replicative gene expression are absent. Furthermore, temperature shift up experiments reveal a defect in intermediate mRNA metabolism in Dts36 infected cells. The results presented in this study indicate that at the non-permissive temperature, the mRNA capping, the early transcription termination, and the intermediate transcription initiation activities of the vaccinia virus capping enzyme are affected in Dts36.

Results

Marker rescue and DNA sequencing

Dts36 and Dts50 temperature sensitive (ts) vaccinia virus mutants comprise a newly discovered complementation group (Lackner et al., 2003). In order to determine the site of the mutation in these viruses, three rounds of a one-step marker rescue were utilized (Kato, Moussatche, Condit and others, accepted for publication). Briefly, confluent monolayers of BSC40 cells were infected with either the Dts36 or the Dts50 virus and transfected with DNA fragments that were PCR-amplified from wild type vaccinia virus DNA. The infected and transfected cells were incubated at 39.7 °C for four days and subsequently stained with crystal violet. A successful rescue of the ts virus is evident from the appearance of plaques on the dishes that were infected and transfected. The first round of marker rescue used PCR products comprising overlapping 20 kb fragments spanning the vaccinia virus genome (Yao and Evans, 2003). The second round of marker rescue used PCR products that used overlapping 5 kb PCR fragments spanning the vaccinia virus genome (Luttge and Moyer, 2005) and the third round used PCR products for individual open reading frames of vaccinia virus genes. Marker rescue of Dts36 (Fig. 1) and Dts50 (data not shown) shows that each mutant was rescued by a PCR product amplified from the wild type D1R gene which indicates that both viruses possess a mutation in D1R, the large subunit of the viral mRNA capping enzyme.

The D1R gene from Dts36, Dts50, and two parental strains of vaccinia virus, IHDW and WR, was sequenced as described in materials and methods. IHDW was sequenced because it is the parental strain from which the temperature sensitive mutants in the study were made and WR was sequenced to determine if there are any differences in the D1R gene between the two parental strains of vaccinia virus. The sequence of D1R in the WR strain was found to be identical to the previously published sequence. By contrast, there are 24 polymorphisms in D1R comparing the WR and IHDW parental strains. Of these 24 polymorphisms, only three encode changes in the protein sequence of D1 (V5I, T202K, and R812K). Both Dts36 and Dts50 were found to have an identical single point mutation at nucleotide residue 2114 which causes a glycine to aspartic acid coding change at amino acid residue 705 in the protein sequence (G705D). Thus, Dts36 and Dts50 are sibling viruses and therefore, for the remainder of this study, only Dts36 was used.

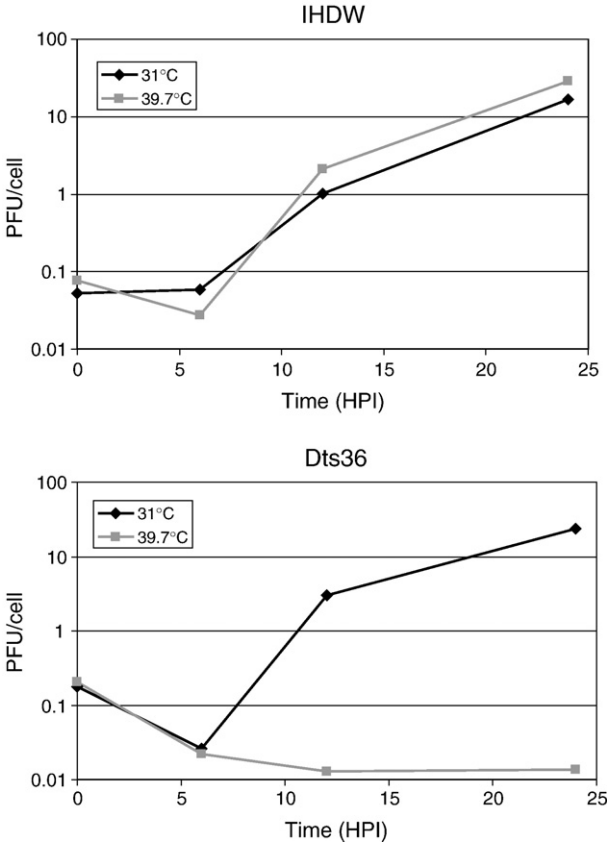

Fig. 2. One-step growth of Dts36 and IHDW viruses. Cells were infected at an m.o.i. of 10 pfu/cell and incubated at 31 or 39.7 °C. Samples were collected at various times post-infection (*x*-axis) and viral yields were determined by plaque titration at 31 °C (*y*-axis). A representative experiment is shown.

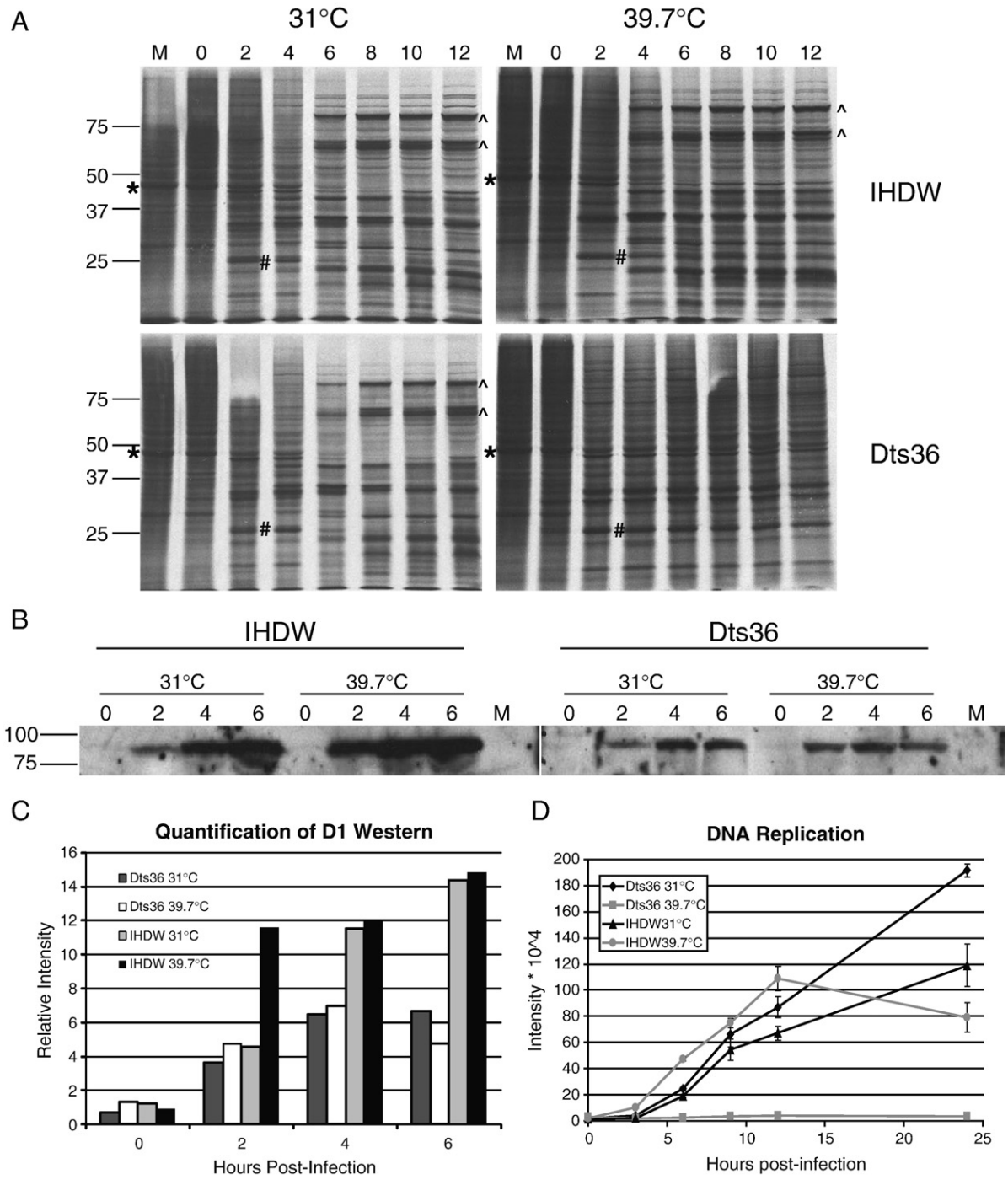

Fig. 3. Protein synthesis, D1 synthesis, and DNA replication in Dts36 and IHDW infected cells. (A) Autoradiograms of protein synthesis in Dts36 and IHDW infected cells. Cells were infected at an m.o.i. of 10, incubated at either 31 or 39.7 °C, and at various times post-infection were metabolically labeled with [^{35}S]methionine for 30 min. The lysates were analyzed on 11% SDS-polyacrylamide gels and the gels were dried and autoradiographed. The virus with which the cells were infected is listed to the right of the autoradiograms and the temperature at which the infected cells were incubated is listed above the autoradiograms. The approximate molecular weight in kDa is listed to the left of the autoradiograms. Examples of the different classes of proteins are marked as follows: *=host protein to the left of each autoradiogram, #=early viral protein between 2 and 4 hpi on each autoradiogram, ^=post-replicative viral protein to the right of each autoradiogram. (B) Western blot analysis of D1 protein in Dts36 and IHDW infected cells. Cells were infected with an m.o.i. of 10, incubated at either 31 or 39.7 °C, whole cells lysates were collected at various times post-infection and analyzed. The virus used to infect the cells, the temperature at which the infected cells were incubated, and the hour-post infection at which the lysates was collected are listed above the autoradiograms. The approximate molecular weights in kDa are listed to the left of the autoradiograms. (C) Quantification of D1 western blot autoradiogram. The hour post-infection for each sample is on the *x*-axis and the relative intensity is on the *y*-axis. (D) DNA synthesis in cells infected with Dts36 or IHDW. Cells were infected with an m.o.i. of 10, incubated for various amounts of times at 31 or 39.7 °C, and analyzed by slot blot. Samples were analyzed in triplicate and the resulting plot of quantified data represents an average of these values. Error bars represent the standard deviations based on the quantified data.

Growth properties of Dts36

A one-step growth experiment was used to determine the growth properties of Dts36 and IHDW. Confluent monolayers of BSC40 cells were infected with a high m.o.i. at 31 or 39.7 °C and infected cells and media were harvested and used to measure viral yield in plaque assays at 31 °C (Fig. 2). At 24 hours post-infection (hpi), IHDW grown at either 31 or 39.7 °C produced between 16 and 29 pfu per cell. Dts36 grown at 31 °C was virtually indistinguishable from IHDW grown at either temperature. However, Dts36 grown at 39.7 °C produced no detectable infectious progeny when analyzed by plaque assays at 31 °C.

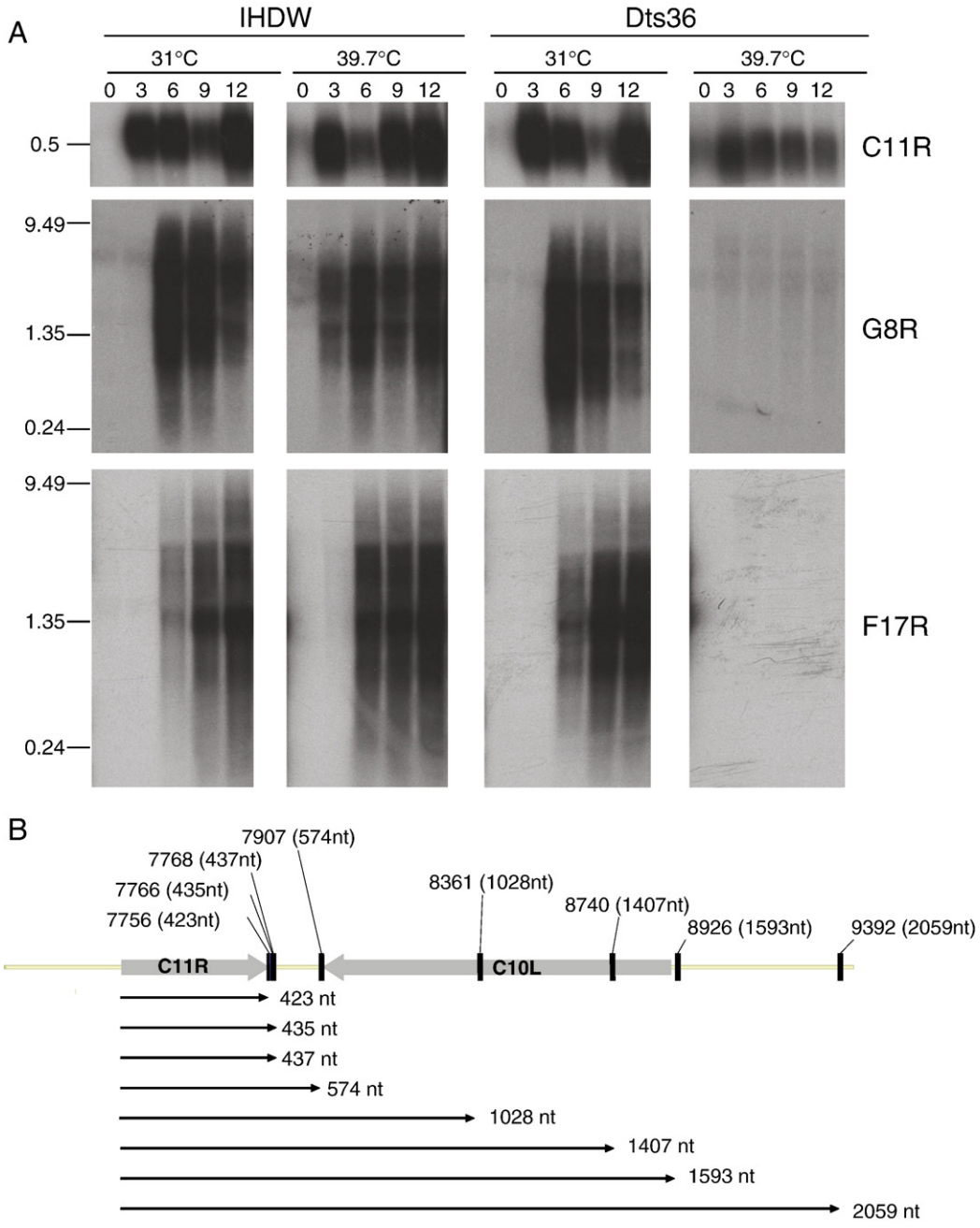

Fig. 4. Analysis of RNA synthesis in cells infected with Dts36 or IHDW. (A) Northern blot analysis on total RNA isolated from cells infected with either Dts36 or IHDW and incubated at either 31 or 39.7 °C were performed. Riboprobes specific for an early gene (C11R), an intermediate gene (G8R) and a late gene (F17R) were used in this analysis. The virus with which the cells were infected, the temperature at which the cells were incubated, and the time post-infection at which the samples were isolated are listed above the autoradiograms. The riboprobe that was used for each northern is listed to the right of the autoradiogram and the approximate molecular weight in kb is listed to the left. (B) A map of the region of the vaccinia virus genome that contains the C11R gene. The C11R gene and the downstream C10L gene are shown as arrows which point in the direction in which they are transcribed. Early transcription termination signals are represented as black boxes. Below the genome map are arrows which represent C11R transcripts that initiate at the C11R initiation site and which would terminate at the different termination signals downstream of C11R. The length of these transcripts is listed at the right end of each transcript.

Basic phenotypic characterization

The basic phenotypic characterization of Dts36 included examining viral protein synthesis, viral DNA replication, and viral mRNA synthesis at the non-permissive temperature. Viral protein synthesis was used as a measure of overall viral gene expression in mutant infected cells. Cells infected with either Dts36 or IHDW at 31 or 39.7 °C were metabolically labeled with ^{35}S methionine, resulting samples were analyzed by SDS-PAGE, and radiolabeled proteins were detected by autoradiography (Fig. 3A). The normal temporal expression of viral genes is evident in the wild type infection. Early viral proteins are visible at 2 h post-infection and their synthesis peaks at 4 hpi when infected cells are incubated at 31 °C and at 2 hpi when infected cells are incubated at 39.7 °C. The shut-off of host cell protein synthesis is also evident in wild type infections as early as 2 hpi and extends throughout the remainder of the infection. Post-replicative protein synthesis is marked by the appearance of two viral proteins, p4a and p4b, beginning at 6 hpi in infected cells incubated at 31 °C and at 4 hpi in infected cells incubated at 39.7 °C and continuing throughout the remainder of the experiment. The pattern of protein expression in Dts36 infections at 31 °C is indistinguishable from IHDW at either temperature. By contrast, Dts36 infections incubated at 39.7 °C showed extended early viral protein synthesis, decreased shut-off of host protein synthesis, and no post-replicative viral protein synthesis. The synthesis of the D1 protein was verified by Western blot analysis and quantified using Image J (Fig. 3B, C). Dts36 synthesized 50–60% less D1 protein compared to IHDW at both temperatures; however, Dts36 synthesized similar amounts of the D1 protein at both the permissive and the non-permissive temperatures, as did IHDW (Fig. 3C).

Viral DNA synthesis in Dts36 and IHDW infected cells was also examined. DNA was isolated from infected cells, applied to a nylon membrane, probed with a radiolabeled probe specific for a region of the vaccinia virus genome, and quantified using a phosphorimager (Fig. 3D). The kinetics of DNA replication in IHDW infected cells at both 31 and 39.7 °C and in Dts36 infected cells at 31 °C were virtually indistinguishable. In non-permissive infections, no viral DNA replication was observed in Dts36 infected cells.

The synthesis of viral mRNA in Dts36 infections at the non-permissive temperature was analyzed by northern blot. RNA was isolated from infected cells incubated at either 31 or 39.7 °C, separated by gel electrophoresis, transferred to a neutral membrane and probed for viral gene expression. The riboprobes used were specific for a gene from each of the three classes of viral genes. Autoradiograms of northerns for the early gene (C11R, the vaccinia growth factor), the intermediate gene (G8R, a vaccinia late transcription factor) and the late gene (F17R, a DNA binding phosphoprotein) are shown in Fig. 4A. In infections with the WR strain of vaccinia virus, early gene expression peaks and then

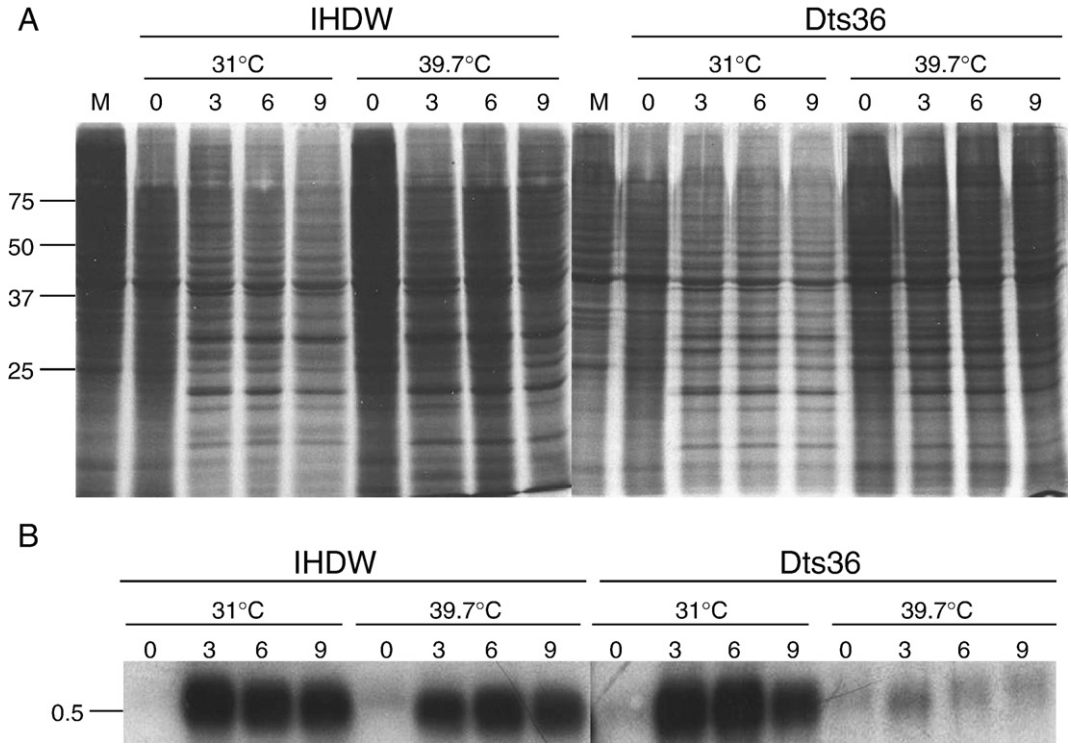

Fig. 5. Viral protein and mRNA synthesis in cells infected with Dts36 and IHDW in the presence of araC. (A) Autoradiograms of protein synthesis in Dts36 and IHDW infected cells in the presence of araC. The infections and analysis of samples were performed. The virus with which the cells were infected, the temperature at which the infected cells were incubated, and the time post-infection at which the samples were isolated are listed above the autoradiograms. The approximate molecular weight in kDa is listed to the left of the autoradiograms. (B) Northern blot analysis using the C11R specific riboprobe was performed. The virus with which the cells were infected, the temperature at which the cells were incubated, and the time post-infection at which the samples were isolated are listed above the autoradiograms. The molecular weights in kb are listed at the left of the autoradiograms.

declines for the remainder of the infection (Baldick, and Moss, 1993). The current study is the first time that gene expression has been examined in IHDW infections and much to our surprise, in wild type IHDW infections, early gene expression peaks, decreases and then resumes at times late in infection. Early gene expression as analyzed by northern analysis for IHDW infected cells at 31 and 39.7 °C and for Dts36 infected cells at 31 °C are virtually indistinguishable from one another. The peak of synthesis of the C11R transcript occurs at 6 hpi at 31 °C for both viruses and at 3 hpi at 39.7 °C for IHDW. The peak of synthesis of C11R transcript in Dts36 at 39.7 °C occurs at 3 hpi as in IHDW at this temperature; however, the mutant produces only 35% the amount of C11R transcript compared to wild type virus infections at this time. The C11R transcript in the Dts36 infection incubated at 39.7 °C also does not reappear at times late in infection. It should also be noted that the C11R transcript in both viruses at both temperatures is of the correct size, approximately 0.5 kb (Fig. 4B). Much like the analysis of C11R gene expression, the intermediate and late gene expression for IHDW infections at 31 and 39.7 °C and for Dts36 infections at 31 °C are virtually indistinguishable from one another; however, the analysis of post-replicative gene expression in mutant infections at 39.7 °C showed no intermediate or late viral transcripts, consistent with the absence of viral DNA replication and post-replicative proteins

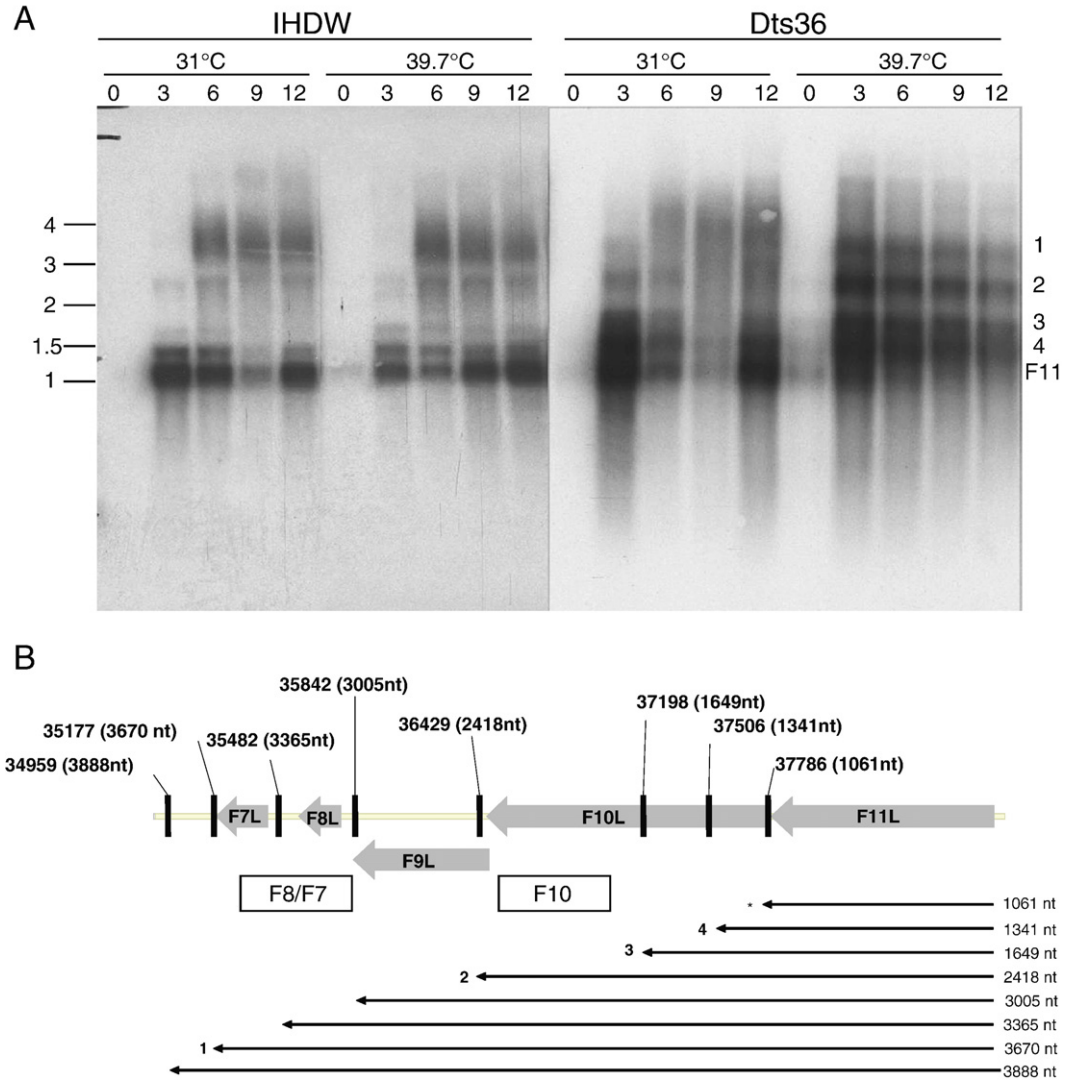

Fig. 6. Analysis of the early viral transcript, F11L, in cells infected with Dts36 or IHDW at either 31 or 39.7 °C. (A) Northern blot analysis of F11L transcripts was performed. The virus with which the cells were infected, the temperature at which the infected cells were incubated, and the time post-infection at which the samples were isolated are listed above the autoradiograms. The approximate molecular weight in kb is listed to the left of the autoradiogram. The F11L transcript is labeled as F11L to the right of the autoradiograms. The longer-than-expected F11L transcripts are also labeled to the right of the autoradiograms. (B) A map of the region of the vaccinia virus genome that contains the F11L gene. The F11L gene and the downstream genes are shown as arrows which point in the direction in which they are transcribed. Early transcription termination signals are represented as black boxes. Below the genome map are arrows which represent F11L transcripts that initiate at the F11L promoter and which terminate at the different termination signals downstream of F11L. The lengths of these transcripts are listed at the right end of each transcript and those transcripts which correspond to bands 1–4 in part A are labeled at the left end of the transcript. The regions of the genome where the F10 and F8/F7 riboprobes hybridize are shown in boxes above the transcripts.

discussed earlier. These results also suggest that the resumption of early gene expression observed in permissive infections requires late gene expression.

Dts36 appeared to synthesize the same amount of early viral proteins as IHDW at the non-permissive temperature when measured by protein pulse labeling (Fig. 3A). However, the lack of host cell protein synthesis shut-off in Dts36 infections incubated at 39.7 °C and the earlier switch to post-replicative protein synthesis in IHDW infections incubated at 39.7 °C made it difficult to accurately compare protein synthesis levels in the two viruses. Therefore, an additional pulse labeling and RNA isolation was performed in the presence of cytosine β-D-arabinofuranoside (araC), an inhibitor of DNA synthesis that would allow accumulation of early viral message and protein (Fig. 5). The pattern of early viral protein synthesis in infections with Dts36 and IHDW at both temperatures was indistinguishable with both viruses apparently synthesizing the same proteins in the same amounts (Fig. 5A). In the analysis of early viral mRNA synthesis, cells infected with IHDW at both temperatures and cells infected with Dts36 at 31 °C were indistinguishable; however, the amount of C11R message at 3 hpi in the Dts36 infections was 15% of that measured in the IHDW infections (Fig. 5B).

Early gene transcription termination is defective in Dts36-infected cells

Analysis of other early viral genes by northern and western blots was performed to investigate whether the observed decrease in steady state early viral RNA is a global phenomenon or if it is specific for the C11R gene. The early viral genes examined were F11L (a gene involved in cell signaling (Valderrama et al., 2006)), E9L (the DNA polymerase), A20R (a DNA polymerase processivity factor (Klemperer et al., 2001)) and D5R (an ATPase/DNA primase (Boyle et al., 2007; De Silva et al., 2007)). Analysis of the F11L transcript showed that similar patterns of expression were evident in infections with IHDW at both temperatures and Dts36 at the permissive temperature (Fig. 6A). In the wild type infections, the major F11L transcript migrates at 1 kb. A second, slightly slower migrating band is seen at 1.35 kb (band 4 in Fig. 6A) and most likely represents termination at the next downstream termination signal past the F11L termination signal. Both bands peak at 3 hpi, decrease, and reappear at late times during the wild type infection at both the permissive and the non-permissive temperatures. At late times during the wild type infection, read through from an upstream late gene, most likely F13L, is evident as a smear from 1 kb to greater than 4 kb. The Dts36 infections at 31 °C are similar to wild type infections; however, there are two differences. First, by visual inspection, the ratio of the 1.35 kb band to the 1 kb band (band 4 to the F11L band in Fig. 6A) seems to be greater in the Dts36 31 °C infections than in the wild type infections. Second, there is an additional band at 2.4 kb in the mutant infections at the permissive temperature indicating that the mutant is slightly leaky at the permissive temperature (see below). The pattern of F11L transcript expression was different in Dts36 infected cells incubated at 39.7 °C. The F11L transcript was present but was no longer the predominant transcript. There were four additional transcripts: band 1 (3.6 kb), band 2 (2.4 kb), band 3 (1.65 kb) and band 4 (1.35 kb) (Fig. 6A).

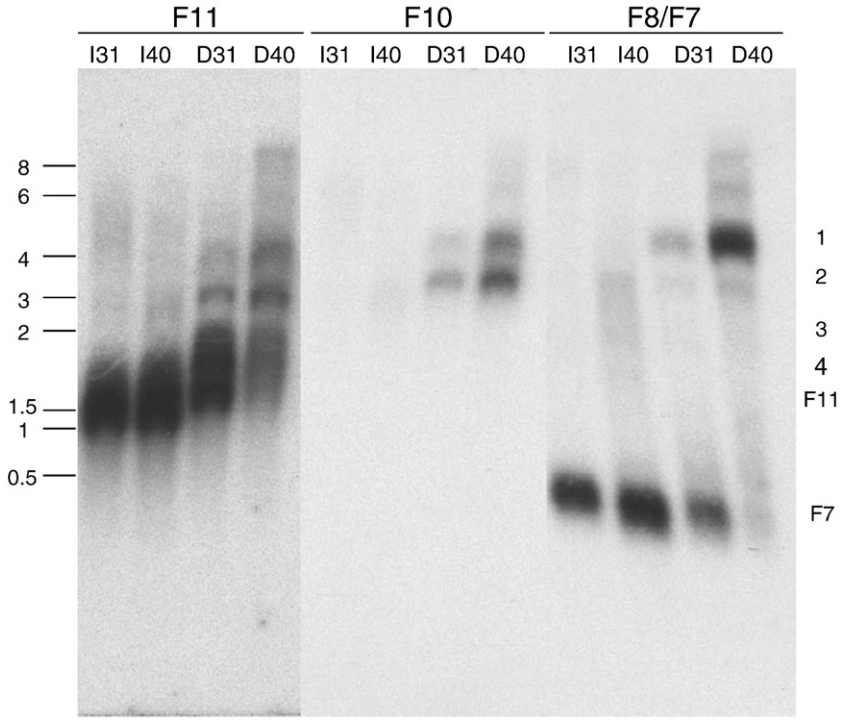

Fig. 7. Primer walk with various riboprobes for analysis of longer-than-expected F11L transcripts. The northern blot analysis was performed. The RNA that was analyzed was isolated at 3 hpi. The riboprobe used in the hybridization and the virus with which the cells were infected are listed above the autoradiograms. I31 = IHDW, 31 °C; I40 = IHDW, 39.7 °C; D31 = Dts36, 31 °C; D40 = Dts36, 39.7 °C. The molecular weights are indicated to the left of the autoradiograms and bands 1–4, the F11L transcript and the F7L transcript are labeled to the right of the autoradiograms.

The F11L transcript and the additional transcripts all peaked at 3 hpi, decreased slowly, and did not reappear at times late during infection. The read through from upstream late genes was also not apparent in mutant infections at the non-permissive temperature. The size of these additional bands corresponds to the predicted length of transcripts that initiate at the F11L promoter and terminate at termination sequences downstream suggesting that these bands result from read-through of termination sequences (Fig. 6B). In order to confirm that these bands were indeed longer-than-expected F11L transcripts, further northern analyses were done using riboprobes designed to detect the longest two additional transcripts seen in the initial F11L northern analysis (Fig. 7). One riboprobe, designated F10, was capable of detecting the two longest of the additional transcripts and a second riboprobe, designated F8/F7, was capable of detecting only the longest additional transcript. The results showed that when the F10

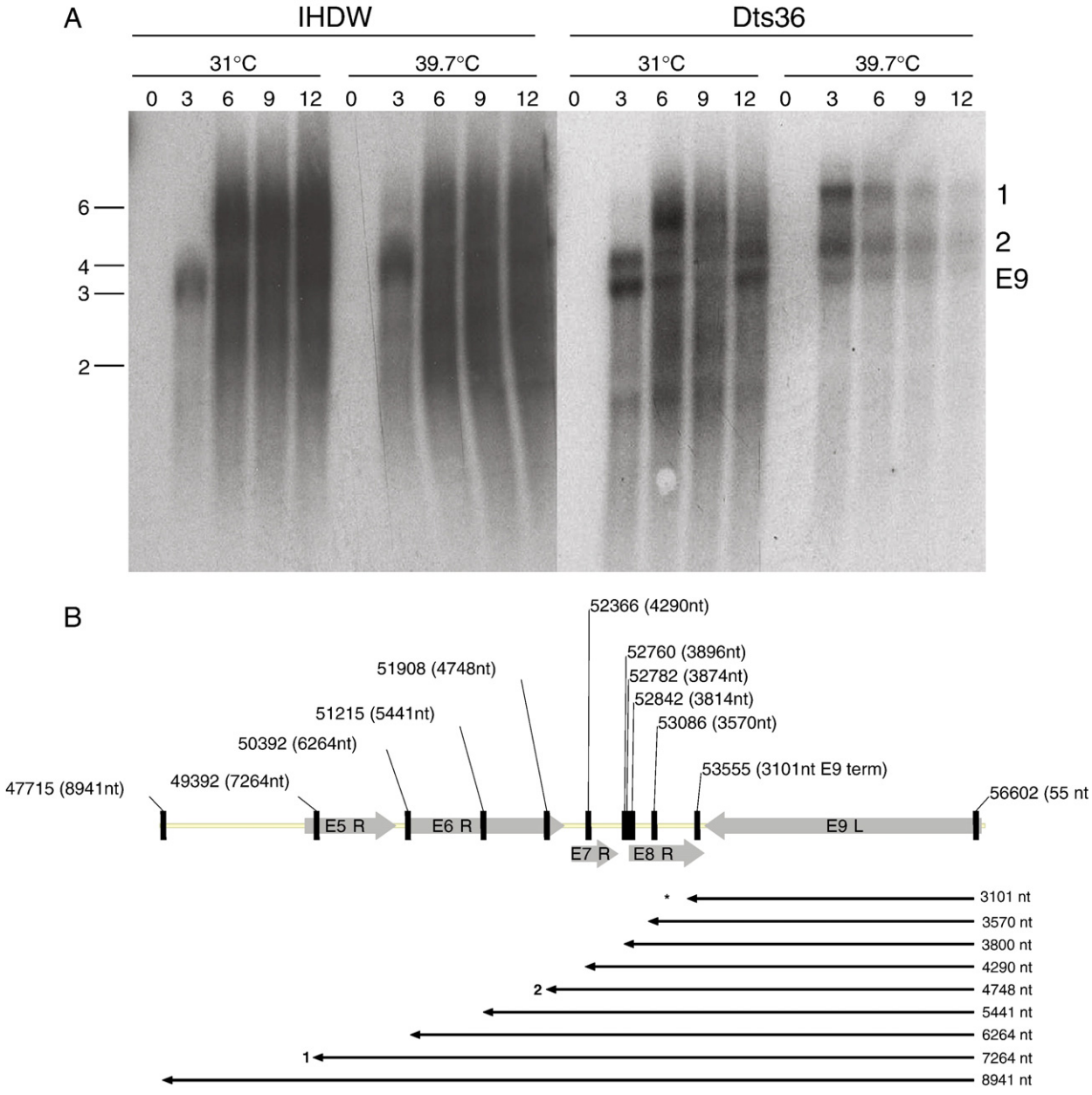

Fig. 8. Analysis of the early viral transcript, E9L, in cells infected with Dts36 or IHDW at either 31 or 39.7 °C. (A) Northern blot analysis of E9L transcripts was performed. The virus with which the cells were infected, the temperature at which the infected cells were incubated, and the time post-infection at which the samples were isolated are listed above the autoradiograms. The approximate molecular weight in kb is listed to the left of the autoradiogram. The E9L transcript is labeled as E9 to the right of the autoradiograms. The longer-than-expected E9L transcripts are also labeled to the right of the autoradiograms. (B) A map of the region of the vaccinia virus genome that contains the E9L gene. The E9L gene and the downstream genes are shown as arrows which point in the direction in which they are transcribed. Early transcription termination signals are represented as black boxes. Below the genome map are arrows which represent E9L transcripts that initiate at the E9L initiation site and which terminate at the different termination signals downstream of E9L. The lengths of these transcripts are listed at the right end of each transcript and those transcripts which correspond to bands 1 and 2 in part A are labeled at the left end of the transcript.

riboprobe was used only the two largest transcripts were seen and when the F8/F7 riboprobe was used only the largest transcript was seen in Dts36 at 39.7 °C. The leakiness seen in permissive infections with Dts36 in Fig. 6 is also apparent in this experiment. In addition to detecting band 1 in non-permissive Dts36 infections, the F8/F7 riboprobe was also able to detect the F7L transcript. This transcript behaved much like the C11R transcript in Dts36 infected cells incubated at 39.7 °C: it was present in decreased amounts compared to the wild type infections at either temperature and the mutant at the permissive temperature.

The analysis of the E9L transcript yielded results similar to those seen in the analysis of the F11L transcript (Fig. 8A). The E9L transcript is visible at 3 hpi in wild type infections at both the permissive and the non-permissive temperatures; however, it is difficult to determine if the transcript reappears late during infections because of the large amount of read through from an upstream late gene (most likely E11L). In infections with Dts36 at 31 °C, the E9L transcript is visible as is an additional, slower migrating band at 4.7 kb (band 2 in Fig. 8A). Both of these transcripts peak at 3 hpi, decrease, and reappear at times late during infection. Much like the wild type infections, Dts36 infections incubated at 31 °C show read through of a late gene that is located upstream of E9L. In mutant infections incubated at 39.7 °C, the amount of the E9L transcript was decreased at 3 hpi, expression of E9L did not resume late during infection, and longer-than-expected E9L transcripts appeared. As with F11L, the size of the additional transcripts corresponded to termination sequences downstream of the E9L termination sequence (Fig. 8B). Northern analyses were also used to examine the viral transcripts encoded by A20R and D5R (data not shown). In Dts36 infected cells at 39.7 °C, the A20R transcript was terminated correctly but was present in decreased amounts and did not reappear late during infections. The D5R transcript in mutant infections at the non-permissive temperature was present in decreased amounts, did not reappear late during viral infection, and did not terminate correctly.

It was of interest to determine the effect that the longer-than-expected transcripts and the decreased amount of transcripts had on viral protein synthesis. Therefore, the synthesis of proteins encoded by these transcripts was analyzed by western blot (Fig. 9). For all proteins examined, the protein of interest was expressed in similar amounts in the wild type infections at both temperatures and the mutant infections at 31 °C. When F11 was examined in Dts36 infected cells at 39.7 °C, the mutant expressed at least the same amount if not more of the protein than IHDW at the same temperature. On the other hand, the E9, A20, and D5 proteins were expressed in decreased amounts in Dts36 infected cells at 39.7 °C compared to IHDW at 39.7 °C.

Intermediate gene transcription is compromised in Dts36-infected cells

The multifunctional vaccinia virus capping enzyme has been implicated in intermediate gene transcription initiation; therefore, it was of interest to determine whether intermediate gene transcription was defective in Dts36 infections at the non-permissive temperature. Because Dts36-infected cells are defective in DNA replication and post-replicative gene expression, a typical time course of infection does not provide any insight into the impact of the mutation on intermediate gene expression. However, we were able to circumvent this problem by performing a shift-up experiment in which the infection was initiated and incubated at 31 °C for 8 h and then shifted to 39.7 °C for the remainder of the experiment (Fig. 10A). Viral protein synthesis was analyzed by pulse labeling (Fig. 10B) and viral DNA replication was assayed by slot blot analysis (Fig. 10C). Before and after the shift to 39.7 °C, similar amounts of post-replicative viral proteins and of DNA replication were seen for both IHDW and Dts36 infections. It is important to note that Dts36 continues both post-replicative protein synthesis and DNA replication.

Viral RNA synthesis was also examined in the shift-up experiment by northern analysis in which autoradiographs were used to qualitatively assess RNA synthesis (Fig. 11A) and phosphorimager analysis was used to quantitatively assess RNA synthesis (Figs. 11B–D). Early and late transcripts were synthesized in similar amounts by both viruses after the shift to 39.7 °C. When an intermediate transcript was analyzed, the IHDW infections showed a constant amount of intermediate synthesis whereas the Dts36 infections showed a striking

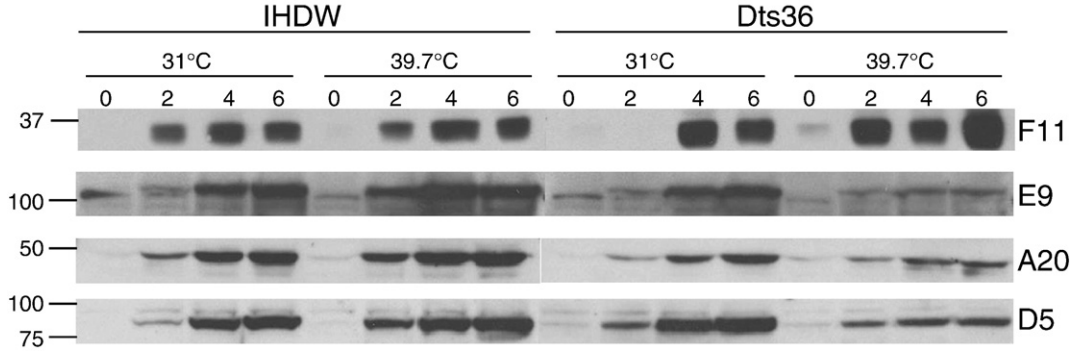

Fig. 9. Synthesis of F11, E9, A20, and D5 proteins during Dts36 and IHDW infections. Samples were analyzed and blots were prepared and probed. The infecting virus, the temperature at which the infected cells were incubated, and the hour post-infection at which the samples were isolated are listed above the autoradiograms. The primary antibody used to probe each membrane is listed to the right of the autoradiograms. The molecular weights in kDa are listed to the left of the autoradiograms.

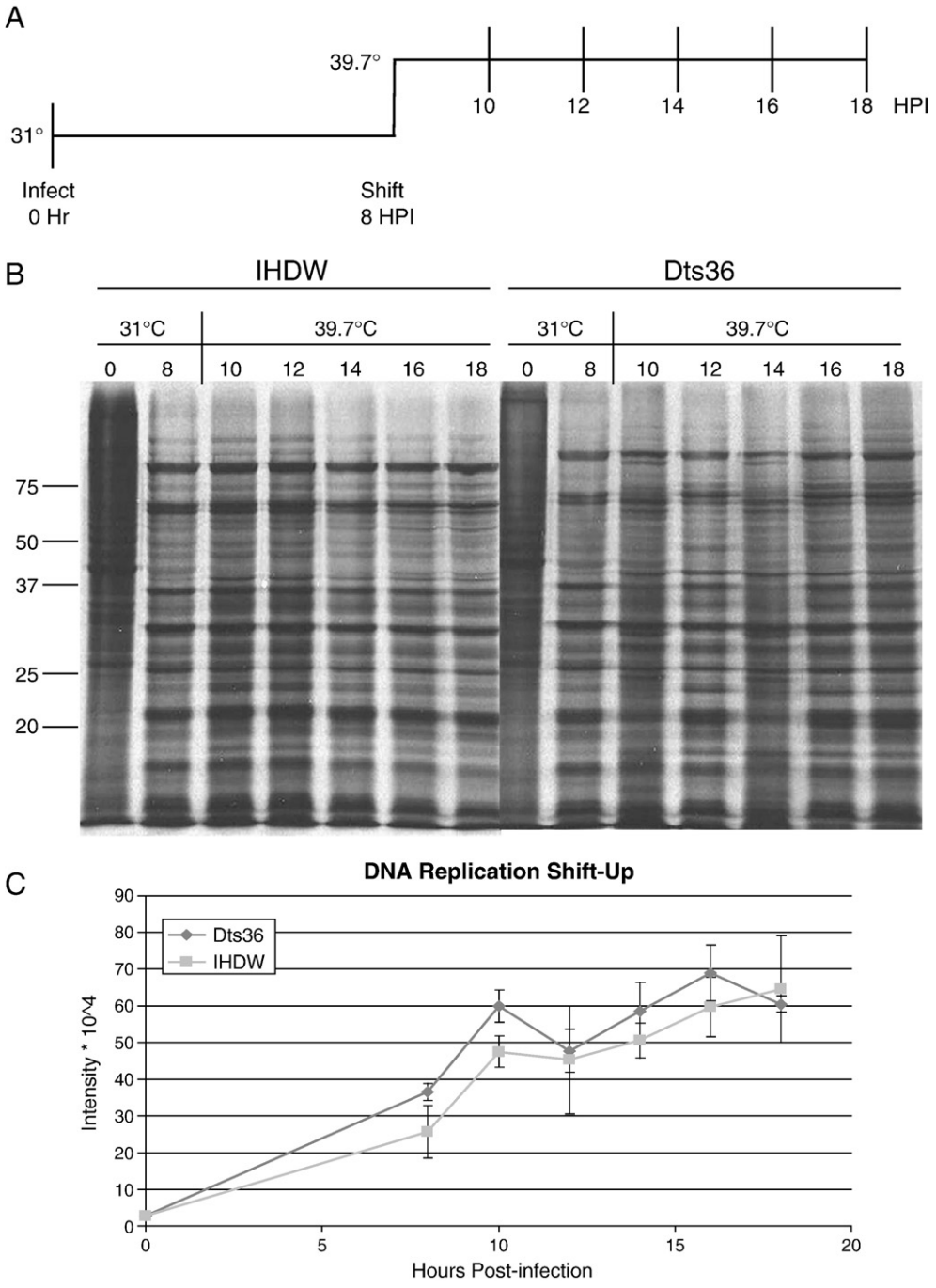

Fig. 10. Analysis of protein synthesis and DNA replication in cells infected with Dts36 and IHDW in a shift-up experiment. (A) Schematic of the shift-up experiment. Cells were infected with either Dts36 or IHDW at an m.o.i. = 10, incubated at 31 °C for 8 h and then shifted to 39.7 °C for the remainder of the experiment. (B) Autoradiograms of protein synthesis in the shift-up experiment utilizing Dts36 or IHDW-infected cells. Samples were analyzed by SDS-PAGE. The virus with which the cells were infected, the temperature at which the cells were incubated, and the time at which the sample was isolated are listed above the autoradiogram. The molecular weights in kDa are shown to the left of the autoradiogram. (C) DNA synthesis in cells infected with Dts36 or IHDW during the shift-up experiment. Samples were isolated and analyzed by slot blot. Samples were analyzed in triplicate and the resulting plot of quantified data represents an average of these values. Error bars represent the standard deviations based on the quantified data.

decrease in intermediate synthesis after the shift to the non-permissive temperature.

Discussion

In this study, the phenotypic characterization of a temperature sensitive mutant in the large subunit of the vaccinia virus mRNA capping enzyme is presented. The detailed analysis of Dts36 showed that this mutant possesses an early phenotype. At the non-permissive temperature, Dts36 infections show aberrant early gene expression, they are DNA negative, and they show no post-replicative gene expression. Analysis of various early mRNA transcripts in Dts36 infections incubated at the non-permissive temperature reveals that some but not all early viral

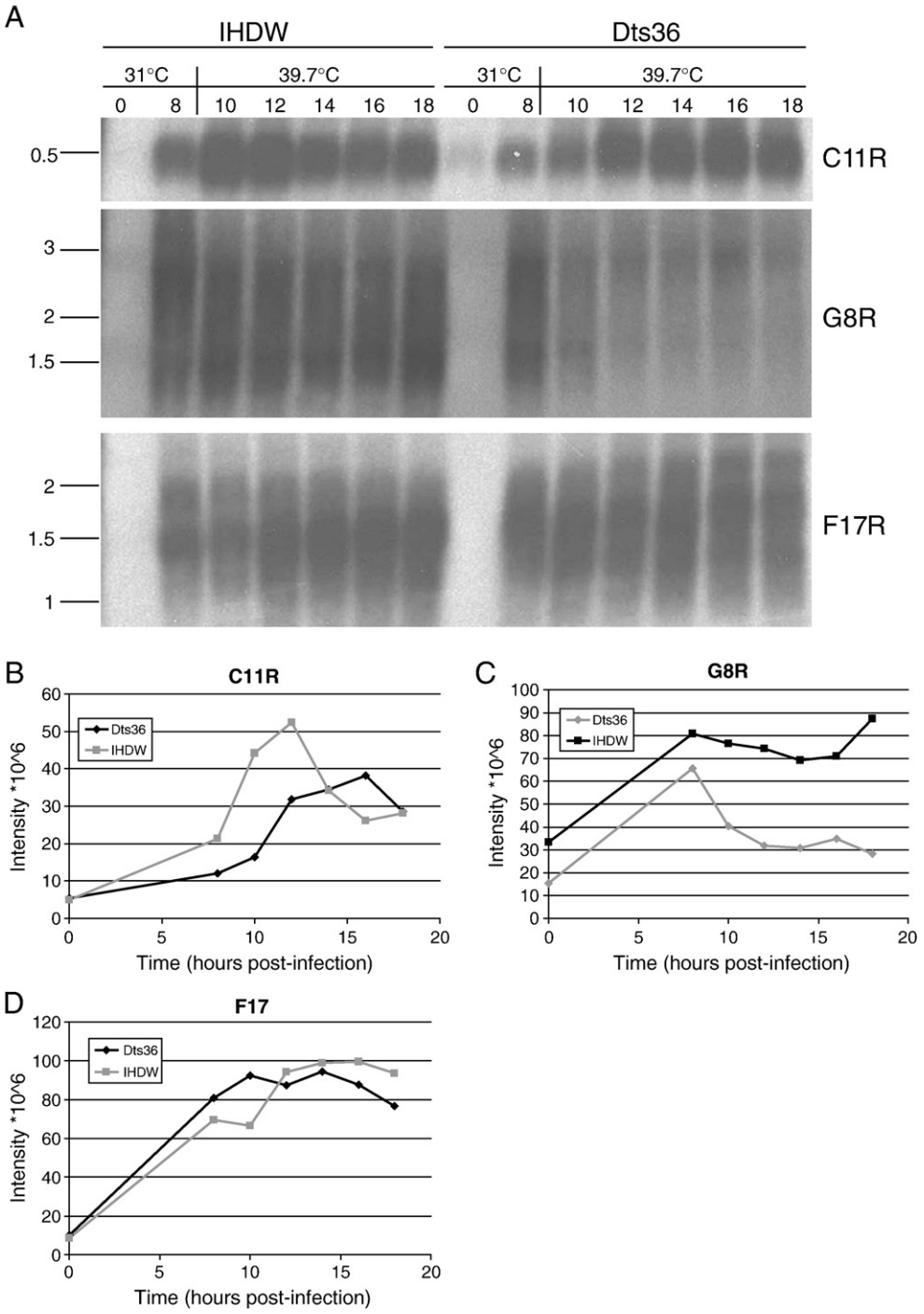

Fig. 11. Analysis of viral mRNA synthesis in cells infected with Dts36 and IHDW in a shift-up experiment. (A) Total RNA was isolated from infected cells and was analyzed by northern blot. The virus used for infection, the temperature at which the infected cells were incubated, and the time when the sample was isolated are listed above the autoradiograms. The molecular weights in kb are listed to the left of the autoradiograms. (B–D) Quantification of viral mRNA synthesis by phosphorimager analysis. The northern blots were exposed to a phosphorimager screen and analyzed (B) C11R, (C) G8R, and (D) F17R. A representative example is shown.

mRNAs are synthesized in reduced amounts. This phenomenon extends to early viral protein synthesis as well. Importantly, we have not measured directly the cap status of viral mRNAs in mutant infected cells. However, given the role of the D1R gene in mRNA capping, the reduction in amounts of both early mRNA and proteins in Dts36 infected cells is consistent with a loss of stability of the nascent mRNA transcripts resulting from a defect in mRNA capping. Additional analysis of early viral mRNA transcripts from infections with Dts36 at the non-permissive temperature also reveals that some early mRNA transcripts are longer than expected. This phenomenon indicates that the mutant capping enzyme encoded by Dts36 is defective in early viral transcription termination at the non-permissive temperature. Finally, analysis of post-replicative

viral transcripts produced during an infection that was initiated at the permissive temperature and then shifted to the non-permissive temperature reveals that the intermediate transcripts decrease in amount after the temperature shift while the late viral transcripts are not affected. This observation is consistent with a defect in the intermediate transcription initiation function of the mutant capping enzyme encoded by Dts36.

mRNA capping

In the current study, Dts36 infected cells were found to synthesize decreased amounts of several early viral proteins at the non-permissive temperature as analyzed by western blot. By contrast, no differences were observed between early viral protein synthesis in wild type and mutant infections as assayed by metabolic labeling. This discrepancy is most likely due to the specificity of each experiment: metabolic labeling focuses predominantly on the most abundantly synthesized viral proteins while western analysis examines steady state levels of specific proteins, regardless of abundance. It may be that for the most actively transcribed genes, mRNA is synthesized in amounts in excess of the translation capacity of the infected cell, so that decreases in the steady state levels of mRNA would not affect the levels of synthesis of the cognate proteins, as assayed by pulse labeling. Several of the proteins that we have analyzed by western blot, notably E9, D5 and A20, may be from genes expressed at relatively lower levels in infected cells. mRNA for these genes may be more limiting during infection so that decreases in steady state levels of active mRNA could have a larger impact on accumulation of the cognate proteins.

In addition to a decrease in amount of some early viral proteins, some early viral transcripts were also reduced in amount in non-permissive Dts36 infections. As noted above, we have not measured directly the cap status of viral mRNAs in mutant infected cells. However, both the decrease in the amount of early viral RNA and protein are consistent with a defect in the ability of the capping enzyme to cap newly synthesized viral transcripts. The defect in the capping activity could represent a specific defect in either the guanylyltransferase or the methyltransferase activity of the mutant enzyme. A defect in the guanylyltransferase activity of the capping enzyme would result in nascent mRNA transcripts that are not guanylylated and therefore, not capped. It has been shown previously that unblocked, i.e. non-guanylylated, reovirus mRNA is less stable in *X. laevis* oocytes, wheat germ extracts and L cell protein synthesizing extracts (Furuichi et al., 1977). Furthermore it has been demonstrated that a decrease in mRNA levels and protein synthesis is observed when mRNA capping activity is inactivated in a yeast guanylyltransferase mutant (Schwer et al., 1998). A defect in methyltransferase activity could affect mRNA function or stability either directly by affecting translation efficiency or indirectly by affecting cap stability. Previous studies in vaccinia virus, reovirus, and vesicular stomatitis virus have shown that methylation of the guanylylate residue of the cap structure of viral transcripts is critical for translation (Both et al., 1975; Muthukrishnan et al., 1975, 1978). Thus a defect in the methyltransferase function of the Dts36 enzyme could result in synthesis of viral mRNA with decreased translation efficiency. A defect in methyltransferase activity could indirectly affect the guanylylated state of the nascent transcript. The guanylyltransferase reaction is reversible in the absence of methylation (Martin and Moss, 1975). Thus a defect in the methyltransferase activity in Dts36 could destabilize the mRNA cap which in turn could lead to degradation of the viral mRNA transcripts and a decrease in viral protein synthesis. As mentioned above, we have been unable to distinguish which, if any, of the two enzymatic activities is defective *in vivo* in the mutant capping enzyme encoded by Dts36. However, we favor the hypothesis that the mutant capping enzyme encoded by Dts36 is defective in the methyltransferase activity, as discussed further below.

Early gene transcription termination

Early gene transcription termination is compromised in Dts36 mutant infections. This represents the first *in vivo* evidence supporting a role for the mRNA capping enzyme in early gene transcription termination, a phenomenon well established *in vitro* (Condit and Niles, 2002). Our data reveals that termination occurs with varying efficiency at some but not all predicted downstream termination signals in Dts36 mutant infections. These observations are consistent with *in vitro* experiments which demonstrate that early viral transcription termination signals are only 75–80% efficient (Earl et al., 1990; Yuen and Moss, 1987). Interestingly the C11R transcript appeared to be terminated properly in Dts36 mutant infections. Analysis of the termination signals of this transcript showed that there are two additional termination signals present within twelve nucleotides of the first termination signal (Fig. 4B). This is consistent with previous *in vitro* studies which predicted that multiple termination signals within a small region enhance termination (Rohrmann et al., 1986; Yuen and Moss, 1986).

Reactivation of early viral messages

The reappearance of early transcripts late during viral infections with IHDW and Dts36 is most likely due to genetic differences between the commonly used wild type strain of vaccinia virus, WR, and the wild type used in this study, IHDW. In vaccinia virus, early transcription factors are encoded by late genes (Gershon and Moss, 1990) and are packaged into newly formed virions late during infection. Although the factors required for early transcription are present late in infection (Wright and Moss, 1989), vaccinia apparently possesses a mechanism that inhibits transcription from early promoters late during infection. It has been hypothesized that this repression of transcription results either from formation of DNA superhelical structures in newly formed virions or association of the viral genome with DNA-binding proteins (Masternak and Wittek, 1996). However, previous studies have shown that a small subset of early promoters, including the vaccinia virus 7.5k promoter, are reactivated at times late during infection with the WR strain of virus (Garces et al., 1993). In the study presented here, both IHDW and permissive Dts36 infections were shown to express early mRNA transcripts at times late in infection for

all transcripts analyzed. We have sequenced numerous IHDW genes and found no significant differences in the promoter regions relative to the WR strain. Therefore, we suspect that the repression mechanism that is present in infections with the WR strain of vaccinia is not present in the IHDW strain of vaccinia virus.

DNA negativity

We believe that the defect in DNA replication observed during Dts36 mutant infections results from the observed generalized decrease in early viral protein synthesis and does not reflect a direct role for the mRNA capping enzyme in DNA replication. As discussed above, western analysis of several early viral proteins involved in DNA replication, A20, D5 and E9, showed that these proteins were present in decreased amounts in Dts36 infected cells incubated at the non-permissive temperature. A previous study has shown that D5 interacts with A20 by yeast 2-hybrid analysis (McCraith et al., 2000). Additional studies of D5 have shown that D5 is capable of oligomerization and more importantly, that this oligomerization is required for ATPase activity (Boyle et al., 2007). The A20 and E9 proteins have also been shown to interact with each other by co-immunoprecipitation (Klemperer et al., 2001). Additional analysis of the A20/E9 interactions have shown that A20 interacts with D4, the uracil DNA glycosylase, and that this dimeric complex interacts with E9 to form a complex which possesses processive DNA polymerase activity (Stanitsa et al., 2006). The reduced amounts of the A20, D5 and E9 proteins in non-permissive Dts36 infections could prohibit the formation of viable DNA replication complexes which would therefore explain the lack of DNA replication in these infections. Alternatively, it may be that one or another of these or another unidentified early viral protein required for DNA replication are normally present in critically limiting amounts, such that a moderate decrease in protein synthesis causes a profound defect in DNA replication.

An intriguing alternative explanation for the DNA negative phenotype of Dts36 relates to the early stages of virion uncoating. Previous studies have shown that vaccinia virus uncoating proceeds in two steps; an initial rapid uncoating after which viral DNA is DNase-resistant and a secondary uncoating after which the viral DNA is no longer DNase-resistant. The second stage of uncoating requires early viral protein synthesis and presumably releases the viral DNA from the core, making it available as a template for DNA replication (Joklik, 1964a,b; Mallardo et al., 2002). The proteins required for secondary uncoating have not been identified. The observed decrease in early viral protein synthesis could theoretically affect the secondary uncoating of the virus particle in non-permissive infections with Dts36. A defect in secondary uncoating would prevent release of viral DNA from the core and a halt of the virus life cycle at the DNA replication stage.

Intermediate gene transcription

Biochemical experiments have shown that the vaccinia mRNA capping enzyme is an intermediate gene transcription initiation factor (Vos et al., 1991). In the shift-up experiment presented in this study, the steady state levels of the G8R intermediate transcript decreased drastically in Dts36-infected cells after a shift to the non-permissive temperature, whereas the steady state levels of the early and late viral transcripts examined did not show a similar decrease. The decrease in steady state levels of mRNA could theoretically result either from a decrease in mRNA synthesis or an increase in mRNA degradation. While we do not know the cap status of postreplicative mRNAs in mutant infections, and while a defect in capping of postreplicative mRNAs could obviously destabilize the RNAs, we would expect such a destabilization to affect all postreplicative RNAs rather than a specific subclass. Since the decrease observed is specific to the intermediate transcript we believe that the intermediate gene transcription initiation function of the capping enzyme is defective in Dts36. This represents the first *in vivo* evidence in support of a role for the vaccinia capping enzyme in intermediate gene transcription initiation.

Subunit association

In Dts36, the large subunit of the mRNA capping enzyme contains a glycine to aspartic acid substitution at residue 705. G705 is conserved in all poxviruses and cellular cap methyltransferases (Schwer and Shuman, 2006) as well as those from Shope fibroma virus and African swine fever virus (Mao and Shuman, 1994). Recent X-ray crystallographic data shows that G705 is located in strand B5 of a seven stranded beta sheet that comprises the conserved core of the enzyme (De la et al., 2007). Thus G705 is not directly involved in the D1–D12 subunit interaction, as was previously suggested based on biochemical data (Schwer and Shuman, 2006). Nevertheless, a triple mutant of the large subunit of the vaccinia virus capping enzyme (G704, G705A, V707A) was shown to have no interaction with the small subunit and no methyltransferase activity when both proteins were synthesized in reticulocyte lysates (Mao and Shuman, 1994). A yeast complementation assay which assesses vaccinia virus methyltransferase activity (Saha et al., 2003) has shown that the G705D mutation is temperature sensitive in yeast (S. Shuman, personal communication). Based on these studies, it is possible that the G705D mutation found in Dts36 affects the structure of the D1 protein in such a way that the two subunits of the capping enzyme are no longer able to associate efficiently at the non-permissive temperature.

The lack of efficient subunit association in the vaccinia virus capping enzyme could affect the methyltransferase activity as well as the early transcription termination and intermediate transcription initiation functions of the enzyme. Inefficient subunit association would directly affect the methyltransferase activity of the enzyme since the small subunit is required to stimulate the intrinsic methyltransferase activity of the large subunit by causing a predicted confirmation change in the methyltransferase active site of the large subunit (Higman et al., 1992; Niles et al., 1994). Both subunits of the capping enzyme have been shown previously to be required for early gene transcription termination (Luo et al., 1995) and intermediate gene transcription initiation (Condit et al., 1996). However, it is

not known if these activities simply require the presence of both subunits or if subunit association is also required. If in fact subunit association is required for both early gene transcription termination and intermediate gene transcription initiation, then a mechanistic explanation that could account for all of the phenotypic features of non-permissive Dts36 infections is a defect in association of the capping enzyme subunits. Future studies with Dts36 will examine the association of the capping enzyme subunits as well as the enzymatic activities of the capping enzyme using biochemical analysis.

Materials and methods

Cells and viruses

BSC40 cells, a continuous line of African green monkey kidney cells, were grown as previously described (Condit et al., 1983; Condit and Motyczka, 1981). The wild type vaccinia virus strain IHDW and the temperature sensitive mutant Dts36 were cultured as previously described (Lackner et al., 2003). One-step growth and plaque titration protocols have been previously described (Condit et al., 1983; Condit and Motyczka, 1981). For all experiments, an m.o.i. of 10 was used unless otherwise noted. The permissive temperature for viral infections was 31 °C and the non-permissive temperature was 39.7 °C.

Viral DNA isolation and DNA sequencing

Viral DNA was isolated from infected BSC40 cells incubated at 31 °C using Qiagen DNeasy tissue kit (Qiagen) as previously described (Lackner et al., 2003). The D1R gene was PCR amplified using primers outside of the open reading frame of the gene to yield a 2750 bp-product. In addition to the external primers, eight internal primers were used for the sequencing reactions on the D1R PCR product. The University of Florida ICBR DNA Sequencing Core Laboratory performed the sequencing reactions.

Marker rescue

One-step marker rescues were performed as previously described (Kato et al., 2004b). Briefly, 60-mm dishes of confluent BSC40 cells were infected at 31 °C with an appropriate m.o.i. as determined by terminal dilution. After infection of the cells, 1.5 μg of DNA PCR-amplified from wild type viral DNA was transfected using Lipofectin reagent (Invitrogen). Infected and transfected cells were incubated at 39.7 °C for four days after which they were stained with crystal violet and the number of wild type plaques were counted.

Protein pulse labeling and gel electrophoresis

Infected monolayers of BSC40 cells were metabolically labeled and the samples were analyzed as previously described with the following modifications (Condit and Motyczka, 1981). Infected cells were labeled with 100 μCi/ml of Redivue Pro-mix$_L$-[^{35}S] *in vitro* cell labeling mix (1000 Ci/mmol) (GE Healthcare) for 30 min. Samples were analyzed by 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After staining with Coomassie blue and subsequent destaining, the gels were dried and exposed to film.

RNA isolation

RNA was isolated from infected cells as was previously described (Cresawn et al., 2007). Briefly, confluent monolayers of BSC40 cells were infected with an m.o.i. of 10 and were incubated at 31 °C or 39.7 °C until RNA was isolated at various times post-infection. Total cellular RNA was isolated using the RNeasy RNA isolation kit (Qiagen) according to the directions of the manufacturer. RNA was eluted from the RNeasy columns with 50 μl RNAse-free water. The concentration of the RNA was determined by measuring the absorbance at 260 nm.

Riboprobes

Antisense riboprobes specific for C11R, G8R, and F17R mRNA transcripts were prepared as previously described (Hassett et al., 1997). The antisense riboprobe for F11R was PCR amplified from a pGEM plasmid containing the open reading frame of the F11R gene linked to the bacteriophage SP6 polymerase promoter. The M13 forward and reverse primers were used for this amplification. Riboprobes specific for approximately 500 base pairs at the 5′ end of A20R, D5R, and E9L mRNA transcripts as well as the primers used in the F11 primer walk experiment (Fig. 7) were transcribed from PCR products as previously described (Cresawn et al., 2007). All riboprobes were transcribed using the MAXIscript *in vitro* transcription system (Ambion) and purified using the NucAway Spin Columns (Ambion) according to the manufacturer's protocol.

Northern blot analysis

Northern blot analysis was performed as previously described with the following modifications (Cresawn et al., 2007). Denaturing formaldehyde agarose gels were run for 2 h at 80 V. Following transfer, the membranes were pre-hybridized for 6 h. Following hybridization the membranes were washed one time at room temperature with 0.1× SSC/0.1%SDS followed by three washes with 0.1× SSC/1.0% SDS at 65 °C. The membranes were exposed to film and then to a phosphor screen (Molecular Dynamics) for quantification of viral RNA transcripts. The quantification data was analyzed using a Storm phosphorimager (Molecular Dynamics) and the ImageQuant software program (Molecular Dynamics).

Western blot analysis

Whole infected cell protein lysates were electrophoresed on 11% SDS-PAGE and transferred to nitrocellulose membranes as described previously (Kato et al., 2004b). Membranes were blocked overnight in a solution of 0.15 M NaCl, 0.05 M Tris pH7.5, 0.1% Tween-20, and 5% nonfat dry milk (TBS-T/NFDM). The membranes were incubated with an appropriate

dilution of primary antibody in TBS-T/NFDM for 1.5 h after which the membranes were rinsed six times with TBS-T. The membranes were then incubated with anti-rabbit Ig conjugated to horseradish peroxidase (Amersham Pharmicia) at a dilution of 1:5000 in TBS-T/NFDM for 1.5 h. The membranes were rinsed six times with TBS-T and developed using the Enhanced chemiluminescence detection kit according to the manufacturer's instructions (GE Healthcare). The antisera used were as follows: anti-E9 (1:500), anti-D5 (1:1000), and anti-A20 (1:1000) rabbit sera were supplied by Dr. Paula Traktman (Medical College of Wisconsin). Anti-D1 (1:500) rabbit serum was supplied by Dr. Ed Niles (SUNY-Bufõ-falo). Anti-F11 (1:2000) rabbit serum was previously described (Kato et al., 2004a). Quantification of autoradiograms was performed using Image J (Abramoff et al., 2004).

Isolation of viral DNA replication samples

Viral DNA was isolated and viral DNA replication was analyzed as described previously (Traktman and Boyle, 2004). Briefly, DNA was isolated from infected cells at various times post-infection by scraping the monolayer of cells into the culture media and recovering the cells by centrifugation. The cells were washed once with phosphate buffered saline (PBS) and resuspended in a solution of 10× SSC (1.5 M NaCl, 0.150 M sodium citrate) and 1 M ammonium acetate. Samples were subject to three cycles of freeze/thawing to disrupt the cells and were stored at −20 °C.

Slot blot hybridization for viral DNA replication

Isolated DNA replication samples were analyzed as previously described (Traktman and Boyle, 2004). The samples were applied to a hydrated and equilibrated Nytran Supercharge nylon transfer membrane (Schleicher and Schuell) on a Minifold II Slot-Blotter apparatus (Schleicher and Schuell). Before removing the membrane from the slot blot apparatus, the DNA was denatured with a solution of 0.5 M NaOH/1.5 M NaCl and then neutralized with two washes of 10× SSC. The membrane was prehybridized at 42 °C in a hybridization oven (Labnet International, Inc) for at least 2 h in a buffer containing 6× SSC, 50% formamide, 0.5% SDS, 5× Denhardts solution (0.1% BSA, 0.1% polyvinylpyrolidone, 0.1% Ficoll), and 100 μg/ml denatured salmon sperm DNA. After pre-hybridization, 2.25×10^6 cpm of the randomly ^{32}P-labeled (DECAprime II kit (Ambion)) HindIII E fragment was added to fresh hybridization solution and incubated with the membranes overnight at 42 °C. The membranes were washed three times with 2× SSC at room temperature followed by two washes with 0.2× SSC/0.1% SDS at 55 °C. The membranes were exposed to film and were then quantified with a phosphor screen (Molecular Dynamics) and analyzed by a Storm phosphorimager (Molecular Dynamics) and the ImageQuant software program (Molecular Dynamics).

Shift-Up

Confluent dishes of BSC40 cells were infected with an m.o.i. of 10 and incubated at 31 °C for 8 h. At 8 h post-infection, the infected dishes were moved to 39.7 °C. At various times post-infection, protein pulse labeling, RNA isolation, and DNA isolation were performed as described above.

Acknowledgments

We thank Paula Traktman and Edward Niles for antisera. This work was supported by NIH grant R01 AI18094 to RCC and by the NIH training grant T32 AI060527.

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