Phenotypic characterization of three temperature-sensitive mutations in the vaccinia virus early gene transcription initiation factor

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Vaccinia virus gene D6R encodes the small subunit of the virion early gene transcription initiation factor. Three temperature-sensitive mutations have been mapped to this gene. The biochemical phenotype exhibited by each mutation was examined. All mutants displayed altered viral protein synthesis in pulse-labelling analyses at both the permissive and non-permissive temperatures. The onset of early protein synthesis was delayed, and the rate of early protein synthesis was reduced in each case. Furthermore the shut-off of both host and early protein synthesis was delayed. In pulse-chase experiments, the stability of the D6R protein in E93- or C46-infected cells was shown to be reduced at 40 °C relative to that at 31 °C. Early mRNA was quantified in cells at 2 h post-infection and shown to be reduced substantially. The ability of each mutant virus to support transcription in vitro was examined at both temperatures and, of the three mutants, only $4 transcription was shown to exhibit reversible temperature sensitivity.

Introduction

Poxviruses are large DNA-containing viruses that replicate in the cytoplasm of infected host cells (Moss, 1990). Vaccinia virus (VV), the best characterized poxvirus, has a dsDNA genome of 191,686 bp (Goebel et al., 1990) and encodes approximately 200 proteins. To carry on their life cycle, poxviruses encode the enzymes necessary to express their genes and replicate their DNA. The enzymes required for early gene expression are encapsidated in the virion core and include a multi-subunit RNA polymerase (Baroudy & Moss, 1980; Nevins & Joklik, 1977), a class-specific trans-acting transcription factor (Broyles et al., 1988; Keck et al., 1990; Vos & Stunnenberg, 1988; Yuen et al., 1987), an early gene transcription termination factor (Rohrmann et al., 1986; Shuman et al., 1987), an mRNA capping enzyme (Martin et al., 1975; Wei & Moss, 1974) and a poly(A) polymerase (Moss et al., 1975). Gene expression is divided into three temporal categories: early genes are transcribed immediately after infection; intermediate genes are transcribed subsequent to the onset of DNA replication and this requires at least two early gene products (Vos et al., 1991); late genes are transcribed after intermediate gene expression and this requires at least three intermediate gene products (Keck et al., 1990).

Early gene transcription takes place in the virus core (Kates & McAuslan, 1967). Initiation requires a sequence-specific dimeric initiation factor that recognizes and binds to the DNA both upstream and downstream from the transcription initiation site (Broyles et al., 1988, 1991; Rohrmann et al., 1986). This factor possesses an NTPase activity, and ATP hydrolysis is required for high levels of transcription (Broyles & Moss, 1988). The energy released is apparently employed in dissociating the initiation factor from the template DNA, which, in the presence of limiting initiation factor, would permit transcription initiation of many genes (Broyles, 1991). The two subunits of the early transcription initiation factor are the products of genes A8L and D6R (Broyles & Fesler, 1990; Gershon & Moss, 1990).

As part of the characterization of the genes in the VV HindIII D fragment (Niles et al., 1986), we have demonstrated that gene D6R is expressed late in the virus life cycle and that two mRNA species can be identified, differing at their 5' ends (Lee-Chen et al., 1988; Lee-Chen & Niles, 1988). Furthermore, three temperature sensitive (ts) mutations have been mapped to gene D6R (Seto et al., 1987; R. C. Condit & E. G. Niles, unpublished data). Mutations C46 and E93 lie within the N-terminal 15% of the protein, whereas S4 maps within the remaining portion of the gene. A genetic approach to investigating the complex mechanism of transcription initiation would complement the biochemical studies currently under way. In this report, we
describe the biochemical phenotypes of these three D6R mutations.

**Methods**

**Cells and viruses.** Wild-type VV WR and ts mutants C46 (Condit & Motyczka, 1981; Condit et al., 1983) and E93 (Ensigner, 1982) have been described. The parent of mutant S4 was isolated by Dr Richard C. Condit as a double mutant, that is both ts and resistant to the S-adenosylhomocysteine analogue sinefungin. The ts lesion in S4 was separated from the drug resistance locus by back-crossing with wild-type virus. Viruses were propagated on BSC40 cells; wild-type virus was grown at 31 °C or 37 °C, and the mutants at 31 °C (Condit & Motyczka, 1981). Virus preparations were routinely tested for retention of the ts phenotype by comparison of their plaque formation ability at both 31 °C and 40 °C. Each mutant phenotype remained stable. Large scale preparation of wild type and mutant virus, propagated at 31 °C in 150 mm dishes, was carried out as described previously (Joklik, 1962). The yield of virus propagated at 31 °C, was routinely 2 × 10^9 to 3 × 10^9 p.f.u./10^7 cells for wild-type virus, E93 and S4, and 5 × 10^8 p.f.u./10^7 cells for C46.

**Immunological techniques.** In pulse-labelling experiments, infected cells were metabolically labelled, in 60 mm dishes, with 100 μCi/ml [35S]methionine. After 30 min the radioactivity was removed, the monolayer was washed two times with medium, and the infected cells were disrupted by the addition of 0.5 ml 1% SDS, 100 mM-mercaptoethanol, 50 mM-Tris–HCl pH 8 and subjected to two cycles of boiling and freezing.

In the pulse–chase experiments, cells were pulsed for 30 min with 100 μCi/ml [35S]methionine at 6 h post-infection, washed as described above, and the chase was initiated by the addition of 1 ml of medium containing 1 mM-methionine. At various times after initiation of the chase, infected cells were washed and disrupted as described above.

Immunoprecipitations were carried out as described (Niles et al., 1989). Antisera were raised to fusion proteins containing a portion of either the VV D1R or D6R protein. A fusion vector in which a segment of gene D6R, from position 7111 (Niles et al., 1989) was linked to the pATH 3 vector. Induction of synthesis of fusion proteins which were isolated by preparative gel electrophoresis followed by electroelution. They were used as antigens in rabbits, as described by Niles & Seto, (1988). The D6R fusion protein contains the C-terminal 85% of D6R, and the D1R fusion protein contains the middle region of D1R, from 19% to 76%. Anti-D8L (Niles & Seto, 1988), and anti-D12L (Niles et al., 1989) antibodies have been characterized. In separate control experiments, each antibody was shown to precipitate at least 90% of its antigen from infected cell extracts. In Western blot analysis, infected cell proteins were transferred to Transblot (Bio-Rad), incubated with one or more antibodies, and either stained after treatment with an alkaline phosphatase-linked second antibody or autoradiographed after incubation with ^125I-protein A.

**Nucleic acid techniques.** RNA was isolated from cells infected for 2 h at either 31 °C or 40 °C by the guanidinium thiocyanate method (Pacha & Condit, 1985). Gene D1R- (nucleotides 601 to 1959) or D12L- (nucleotides 12858 to 14323) specific dsDNA probes were employed in the Northern blot analyses (Niles et al. 1989). In vitro transcription was carried out in NP40-permeabilized virions prepared as described (Shuman & Moss, 1989). Reactions were conducted at 31 °C or 40 °C, 0.05 absorbance units (at 260 nm) of virus being used in each reaction.

**Results**

**Protein synthesis in virus-infected cells**

The synthesis and stability of viral proteins were assessed in cells infected with wild-type or ts mutant virus at the permissive and non-permissive temperatures. Infected cells were pulse-labelled at various times after infection; cells pulsed at 6 h post-infection were chased for up to 4 h, extracts were prepared, and proteins were separated by gel electrophoresis and observed by autoradiography. In the case of wild-type virus at 31 °C (Fig. 1a), the rate of early protein synthesis peaked at 2 h post-infection and then decreased over the next 6 to 8 h. Late proteins, p4a and p4b, were initially detectable at 4 h but were prominent at 6 h and their synthesis continued throughout the infection. Host protein synthesis was shut off by 4 to 6 h. At 40 °C (Fig. 1b), the rate of early protein synthesis peaked within 1 h and then declined rapidly. Late protein synthesis was readily observed by 4 h and continued throughout infection. The shut-off of host protein synthesis was complete by 4 h post-infection (Moss & Salzman, 1968; Salzman & Sebring, 1967). At both 31 °C and 40 °C, the late structural protein precursors, p4a, p4b and p28 (Katz & Moss, 1970; Moss & Rosenblum, 1973) were processed into their smaller products.

Modest changes in the synthesis of host and E93 viral proteins can be observed at both the permissive and non-permissive temperatures, (Fig. 1c, d). Early protein synthesis began within 1 h but the shut-off of early protein synthesis was delayed in comparison to that in wild-type virus-infected cells. Likewise, the shut-off of host protein synthesis was noticeably delayed in cells infected with E93. Furthermore, there was a delay in the appearance of late proteins p4a and p4b at each temperature, although their processing appeared to be unaffected. In cells infected with S4 at 31 °C, the pattern of protein synthesis was similar to that observed for E93 (Fig. 1e) and at 40 °C, the shut-off of both early viral protein and host protein synthesis, and the initiation of late protein synthesis were noticeably delayed (Fig. 1f). As observed in E93-infected cells, the proteolytic processing of the structural protein precursors proceeded normally during the S4 infection. In the case of C46, the phenotype was even more pronounced with the shut-off of both host and early viral protein synthesis delayed at each temperature (Fig. 1g, h). The onset of late viral protein synthesis was markedly delayed, being apparent only at 8 to 10 h post-infection at the permissive temperature and almost undetectable at 40 °C.

To obtain a more precise model of early and late protein synthesis and early gene expression shut-off, antibodies directed against the early protein D1R, the
late protein D8L (Niles & Seto, 1988) and the D6R protein were used to precipitate the three proteins simultaneously from cells pulse-labelled or chased at 31 °C or 40 °C (Fig. 2a to h). The anti-D6R antibody was characterized by identifying the proteins precipitated from cells pulsed at different times during infection. In Fig. 3, there are three proteins in the immunoprecipitate, a major component of about 72K and two minor proteins of about 66K and 62K. The appearance of one or two minor proteins varied from preparation to preparation and probably resulted from a variable degree of proteolysis of the 72K protein. Note that in Fig. 2(a to h) the rate of synthesis and the turnover of the two visible D6R proteins are equivalent.

In the case of wild-type virus-infected cells, at 31 °C early protein D1R appeared at 1 h and continued to be synthesized throughout the infection. The late protein D8L was the first visible 4 h post-infection. D6R, previously identified as a late gene (Lee-Chen et al., 1988; Lee-Chen & Niles, 1988) encodes two proteins which appeared at about 4 h. At 40 °C, D1R synthesis peaked at 1 h post-infection and its rate of synthesis declined rapidly. D8L was first identified at 4 h and was synthesized throughout infection. The two D6R bands were first seen at 2 h post-infection, before the appearance of D8L, suggesting that D6R may be an intermediate gene.

In the case of E93, at 31 °C (Fig. 2c) the pattern of synthesis of D1R, D6R and D8L was similar to that of the wild-type viral proteins, but was reduced in rate. At 40 °C (Fig. 2d), D1R synthesis was delayed in relation to that of the wild-type viral protein, and its synthesis was not shut off. The synthesis of both D6R and D8L was delayed 2 h. In S4-infected cells, (Fig. 2e,f) the synthesis of the early protein D1R was both delayed and reduced in amount; shut-off did not occur at either temperature. The synthesis of both D8L and D6R was delayed 2 h at each temperature.

Marked alterations in the synthesis of viral proteins were observed at both temperatures in the case of C46 (Fig. 2g,h). D1R synthesis was dramatically delayed and greatly reduced, but shut-off did not occur. The appearance of D6R was delayed at least 2 h and that of D8L by 2 to 4 h.

There appears to be a gradient in the severity of the ts phenotype for both the pattern of total virus protein synthesis, and that of these three specific viral proteins. E93 is most similar to the wild-type virus product, S4 is more distinct and C46 is the most distinct. To quantify more precisely the levels of early viral proteins at both 1 and 2 h post-infection, simultaneous immunoprecipitation using antibodies against the two early proteins D1R and D12L (Niles et al., 1989) was carried out (Fig. 4a to d). It can be seen that at both temperatures each mutant showed reduced levels of synthesis of both early proteins. At 40°C, not only were the levels of each protein reduced, but the time of synthesis was delayed in comparison to that in wild-type virus-infected cells.

Stability of D6R in virus-infected cells

To assess the relative stability of wild-type and ts mutant D6R proteins, pulse-chase analyses were carried out (Fig. 2a to h, lanes C1 to C4). The rate of turnover of D6R was compared to that of the stable late protein D8L, which resides in the membrane of the intracellular form of the virus (Niles & Seto, 1988) or, in the case of C46, to that of the early protein D1R. In wild-type virus-infected cells there was little degradation of D8L and modest turnover of the D6R protein (Fig. 5). In the case of E93 and S4, D8L was stable at both temperatures, but in E93-infected cells the D6R protein was dramatically destabilized at 40 °C (Fig. 5). In the case of C46, because the onset of late gene expression was delayed, we compared the stability of D6R to that of the early protein D1R. In this case, although the D1R protein was stable at both temperatures, the D6R protein was degraded rapidly at the non-permissive temperature. Again, in terms of relative stability, there was a gradation in severity: wild type > S4 > E93 > C46.

Level of virion D6R protein

The reduced rates of early gene expression by the mutant viruses could be explained by a reduced level of the D6R protein being packaged into the progeny virus at 31 °C. To obtain a qualitative assessment of the relative level of D6R in wild-type and ts mutant virus, virus purified through sucrose gradients was subjected to Western blot analysis. Antibodies against both D6R and D8L were simultaneously employed so that the D8L protein could serve as an internal standard (Fig. 6). The level of D6R relative to that of D8L was about 55% 40% and 110% for E93, S4 and C46, respectively, in comparison to the wild-type virus. However, in the case of S4, D6R appeared to be distributed over two protein bands. The relationship between these two proteins is not clear. Note that a low level of the upper protein is observable for both wild-type virus and E93.

Quantification of the level of early mRNA

To determine whether the reduced rates of early protein synthesis observed in the pulse-labelling analyses were due to reduced levels of early mRNA, Northern blot analysis was carried out on total RNA isolated from cells infected with wild-type or mutant virus at 2 h post-infection. Total RNA (5 to 15 µg) was separated by agarose gel electrophoresis and, after transfer, filters were blotted with probes to either early gene D1R (data
Fig. 1. Protein synthesis in wild type and ts mutant VV-infected cells. BSC40 cells were infected at a multiplicity of 20, infected cells were pulse-labelled for 30 min with 100 μCi/ml [35S]methionine, washed and lysed in 2% SDS and 100 mM-2-mercaptoethanol. An aliquot of each lysate was separated by gel electrophoresis and observed by autoradiography. (a, b) Wild-type virus; (c, d) E93, (e, f) S4
Vaccinia virus transcription ts mutations

and (g, h), C46 at 31 °C and 40 °C, respectively. Lanes M, mock-infected cells; lanes 1, 2, 3, 4, 6, 8 and 10, pulse at 1, 2, 4, 6, 8 and 10 h post-infection; lanes C1 to C4, chase of 1, 2 or 4 h; H, host protein; E, early viral protein; p4a, p4b and p28, late viral protein precursors; 4a, 4b and 25K, viral protein processing products.
Fig. 2 Immunoprecipitation of viral proteins from pulse-labelled virus infected cells. An aliquot of each extract, prepared as described in Methods was incubated with antisera raised to fusion proteins containing a portion of the products of genes D1R, D6R and D8L. Precipitates were collected, washed, solubilized in protein gel sample buffer, separated by gel electrophoresis and fluorographed. (a, b)
Wild-type virus- (c, d), E93- (e, f), S4- and (g, h), C46-infected cell extracts at 31 °C and 40 °C, respectively. Lanes W, M, markers; lanes M, extract prepared from mock-infected cells; Lanes 1, 2, 4, 6, 8 and 10, pulse labelling 1, 2, 4, 6, 8 and 10 h post-infection; lanes C1, C2 and C4, cells pulsed at 6 h and chased for 1, 2 or 4 h respectively. P, A late viral protein unrelated to D6R precipitated by the D8L preimmune serum.
Fig. 3. Immunoprecipitation of viral proteins from infected cell extracts, pulsed at 2, 4, 6, 10 and 12 h (lanes 2, 4, 6, 10 and 12) using the anti-D6R antiserum; lane PI, extract from cells pulse-labelled at 6 hours post-infection was treated with preimmune serum. P', An early viral protein unrelated to D6R precipitated with the preimmune serum. Mrs of the D6R-related proteins are shown to the right. Lane W, Mr markers; lane M, mock-infected cells.

not shown) or D12L mRNA (Fig. 7). For E93 and S4, the concentration of mutant D12L mRNA was dramatically reduced in comparison to that of the wild-type virus at both the permissive and non-permissive temperature, demonstrating that the decreased rates of protein synthesis were due to decreased levels of early mRNA. The results observed with the gene D1R probe were identical to those with the gene D12L probe.

Transcription in NP40-permeabilized virions

To determine whether transcription is temperature-sensitive in any of the ts mutants, the rate of transcription in NP40-permeabilized virions was assessed (Fig. 8). Identical amounts of virus were used in each assay. In the case of E93, transcription was at least as efficient as that of wild-type virus at each temperature; C46 exhibited reduced transcription but no indication of temperature sensitivity was observed. However, S4,
Vaccinia virus transcription ts mutations

Fig. 5. Stability of D6R in wild-type (a) or E93 (b), S4 (c) or C46 (d) virus-infected cells. Cells infected at 31 °C (open symbols) or 40 °C (filled symbols) with wild-type or ts mutant virus were pulse-labelled at 6 h post-infection for 30 min. Medium containing an additional 1 mM-methionine was returned to the washed monolayer and at various times after the chase was started, cell extracts were prepared. The levels of D1R (△, ▲) D6R (○, ●) and D8L (□, ▼) were quantified by densitometry and plotted as a percentage of the protein present in the 6 h pulse.

![Fig. 5](image)

Fig. 6. Western blot analysis of D6R and D8L proteins present in wild-type (lane 1), E93, S4 and C46 (lanes 2 to 4) virions. Virus was purified from cells infected at 31 °C, and 0.2 A260 units of virus was disrupted by boiling in 2% SDS and 100 mM 2-mercaptoethanol and separated by gel electrophoresis. The proteins were transferred to Transblot (Bio-Rad) and incubated with a 1:100 dilution of antiserum raised against the D8L and D6R proteins. The positions of the bound D6R and D8L antibodies were determined by incubating with alkaline phosphatase-linked secondary antibody and staining. Equivalent results were obtained for incubations carried out with antisera diluted at either 1:100 or 1:200.

![Fig. 6](image)

Fig. 7. Northern blot analysis of D12L mRNA levels in cells infected for 2 h at 31 °C or 40 °C with wild type (a) or E93 (b), S4 (c) or C46 (d) mutant virus. RNA was prepared from cells infected at a multiplicity of 20 for 2 h at either 31 °C or 40 °C. Total RNA (5, 10 or 15 μg, lanes 1 to 3) was separated by gel electrophoresis and transferred to GeneScreen Plus (NEN). Identical filters were incubated with radioactive DNA probes complementary to D1R or D12L mRNA. After washing, the filters were autoradiographed and the RNA levels were quantified by densitometry. After densitometry, the relative level of D12L mRNA was estimated from the slope of a line derived from a plot of absorbance versus RNA concentration and expressed as absorbance/10 μg total RNA (values at bottom).

![Fig. 7](image)
Fig. 8. Transcription activity in NP40-permeabilized wild-type (a), E93 (b), S4 (c) or C46 (d) virions. Purified virus (0.05 A$_{260}$ units) was permeabilized by treatment with 0.05% NP40 and incubated in the presence of rNTPs including [32p]GTP at either 31 °C (○) or 40 °C (●). At various times after initiating the reaction, an aliquot was removed, and RNA was precipitated with TCA and counted in a liquid scintillation counter. (e) The transcription rate in S4 virions was measured in a temperature shift-down experiment. A transcription assay mixture was first incubated at 40 °C and RNA synthesis was measured at the times indicated. After 10 minutes (↓), one sample was shifted to 31 °C and RNA synthesis was quantified. (○) 31 °C; (●) 40 °C; (Δ) 40 °C shifted to 31 °C.

Fig. 9. Thermolability of the virion transcription machinery. NP40-solubilized wild-type (○), E93 (●), S4 (△) or C46 (□) mutant virus was incubated at 45 °C in transcription assay mix lacking rNTPs. At various times an aliquot was transferred to an in vitro transcription mixture. After incubation at 31 °C for 30 min, an aliquot was removed, and the product RNA was precipitated with TCA and quantified by liquid scintillation counting. The results are presented as the percentage activity remaining in comparison to that of virus not incubated at 45 °C.

Discussion

The ability of each mutant to synthesize mature viral proteins was assessed by pulse-chase analysis. In each case, the mutants exhibited altered viral gene expression in the form of reduced rates of early and late protein synthesis at both the permissive and the non-permissive temperatures. In addition, a marked delay in the expression of both early and late genes is seen at 40 °C. Shut-off of early viral protein synthesis is relatively slow at 31 °C in both the mutant- and the wild-type virus-infected cells. At 40 °C, shut-off proceeds rapidly in wild-type virus-infected cells, but is markedly delayed for each mutant. There is a gradient in severity of the observed phenotype, in which E93 is most similar to wild-type virus, S4 is more severely inhibited and C46 is the most markedly affected.

In several VV ts mutants, the altered protein exhibits an enhanced rate of turnover at the non-permissive temperature (Evans & Traktman, 1987; Hooda-Dhingra et al., 1990). We assessed the rate of turnover of the D6R protein by pulse–chase analysis. E93 and C46 display an elevated rate of degradation at 40 °C, relative both to the wild-type D6R protein and to an internal control, either D8L or D1R. A gradient of stability was observed in which wild-type protein is most stable followed by those of S4, E93 and C46. One possible explanation for the reduced rate of gene expression observed at 31 °C is that owing to a low level of proteolysis of D6R at 31 °C, fewer D6R molecules are packaged into the virus. To obtain a qualitative measurement of the amount of D6R in wild-type and mutant virus grown at 31 °C, the level of D6R was estimated relative to that of another virion protein,
D8L. C46 was shown to possess a level of D6R equivalent to that of the wild-type virus, whereas that of both E93 and S4 was reduced by about 50%. It should be noted that in S4 there are two D6R bands and it is not clear what the relationship between the two components is.

To determine whether the reduced rates of early viral protein synthesis observed in mutant virus-infected cells is reflected in altered mRNA concentrations, the level of early D1R and D12L mRNA was measured by Northern blot analysis at 2 h post-infection and compared to the rate of synthesis of D1R and D12L determined by pulse-labeling. In each case, the mutant virus-infected cells possess less early mRNA then wild-type virus-infected cells. A gradient in severity was again observed in which wild-type > C46 > E93 > S4. For both wild-type virus- and E93-infected cells, the rate of D1R and D12L protein synthesis is roughly proportional to the level of message. In C46-infected cells, the rate of protein synthesis is somewhat less than expected for the amount of early mRNA observed. In the case of S4 infected cells, early mRNA is barely detectable, less than 1% of normal, whereas early proteins are made at 10 to 30% of the wild-type rate. This unusually low level of early mRNA found in S4-infected cells may be due either to a reduced rate of transcription or to an enhanced rate of degradation. It is surprising that in S4-infected cells such a low level of mRNA can allow the synthesis of early proteins at a relatively high rate. This may indicate that early mRNA is produced in excess in vaccinia virus-infected cells.

In an attempt to determine whether transcription is directly affected by any of the mutations, the rate of viral transcription was measured in vitro in NP40-permeabilized virions (Shuman & Moss, 1989). At 31 °C, E93 displayed a transcription rate 25% greater than that of wild-type virus, whereas C46 showed 43% of the wild-type virus transcription rate. For wild-type virus, E93 and C46, the transcription rate is increased at 40 °C demonstrating that RNA synthesis is not temperature-sensitive. However, in the case of S4 the rate of transcription, which is reduced by 25% at the permissive temperature, is decreased to 10% of the wild-type virus level at the non-permissive temperature. The temperature sensitivity of transcription exhibited by S4 was shown to be partially reversible. S4 is the first VV mutation displaying marked viral RNA synthesis temperature sensitivity (Ensinger, 1987; Hooda-Dhingra et al., 1989, 1990).

Within this complementation group there may be two mechanisms conferring temperature sensitivity. The E93 and C46 D6R protein is rapidly turned over in cells infected at 40 °C. This increased rate of degradation would cause reduced levels of D6R to be packaged into progeny virus. As a result, in the next round of replication, early gene expression would be reduced and the infection would be inhibited, preventing plaque formation. Alternatively, since D6R is a component of the virus core, a low level of D6R may result in the formation of an unstable structure. However, the proteolytic processing of the virion structural protein precursors p4a, p4b and p28 proceeds normally in E93-infected cells. Since the cleavage of these precursors is a late step in the virion assembly pathway, it is likely that the alteration in the D6R protein does not affect virion assembly. However, in S4-infected cells the D6R protein is relatively stable at 40 °C. In this virus, in vitro transcription is temperature-sensitive in permeabilized virions and the level of early mRNA in infected cells is dramatically reduced. In the case of S4, ts plaque formation is likely to be due to the presence of an early transcription factor compromised in its ability to support transcription. It is clear from a comparison of the level of early D12L mRNA to the observed rate of D12L protein synthesis that the phenotypes exhibited by these mutations are not straightforward in any case. As a result, the proposed models of temperature sensitivity should be viewed with a degree of scepticism until further experimentation is carried out.

In the case of mutations in the D6R complementation group, as has been shown for ts mutants in the large viral RNA polymerase subunits (Ensinger, 1987; Hooda-Dhingra et al., 1989, 1990), there is a wide range of biochemical phenotypes. The level of viral gene products produced during infection and the timing of their synthesis is affected at both the permissive and the non-permissive temperature. However, there is little obvious correlation between the rate of protein synthesis and the plaque forming ability of the virus. In fact, for C46 and S4 a more dramatic alteration in gene expression is observed at the permissive temperature than is seen for E93 at 40 °C. However, C46 and S4 form plaques at 31 °C, but E93 does not at 40 °C. It is clear that the ability of a virus to form plaques at either temperature is not dependent solely on the amount of viral protein synthesized within the first 10 h of infection. Perhaps, rather than the absolute amount of viral protein synthesized during the infection, it is the timing of gene expression that may be more critical. At 40 °C, the virus life cycle proceeds much more rapidly than at 31 °C. At the higher temperature, alteration of the time of synthesis of one or more viral gene products may be crucial to the successful completion of the life cycle, resulting in ts plaque formation. At 31 °C, the virus life cycle proceeds more slowly and a delay in the appearance of a viral protein may not be critical.

The three mutations provide genetic access to different biochemical features of early gene expression. Although the in vitro transcription rate of E93 is
equivalent to that of wild-type virus, in vivo early mRNA levels are substantially lower than those in wild-type virus-infected cells. C46 exhibits reduced transcription in vitro, but transcription is clearly not temperature-sensitive. Again, in C46-infected cells, D12L mRNA is substantially reduced in vivo. The mechanism responsible for the reduction in early mRNA in E93- and C46-infected cells should be defined. S4 is the most interesting mutation. Since transcription in vitro is reversibly temperature-sensitive, an evaluation of the mechanism of temperature sensitivity would provide insight into the pathway of early gene transcription initiation. Furthermore, the level of early S4 mRNA in vivo is at the limit of detection, less than 1% of that in wild-type virus-infected cells. An analysis of mRNA metabolism in S4 infected cells may provide a unique insight into the pathway of early viral mRNA synthesis and degradation.

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Vaccinia virus transcription ts mutations


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