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Temperature-Sensitive Mutants with Lesions in the Vaccinia Virus F10 Kinase Undergo Arrest at the Earliest Stage of Virion Morphogenesis

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Vaccinia virus encodes two protein kinases; the B1 kinase is expressed early and appears to play a role during DNA replication, whereas the F10 kinase is expressed late and is encapsidated in virions. Here we report that the F10 kinase gene is the locus affected in a complementation group of temperature-sensitive mutants composed of *ts*15, *ts*28, *ts*54, and *ts*61. Although these mutants have a biochemically normal phenotype at the nonpermissive temperature, directing the full program of viral gene expression, they fail to form mature virions. Electron microscopic analysis indicates that morphogenesis undergoes arrest at a very early stage, prior to the formation of membrane crescents or immature virions. An essential role for the F10 protein kinase in orchestrating the onset of virion assembly is implied.

The vital role played by protein phosphorylation in the temporal and stimulus-regulated control of biological processes is increasingly apparent. The orderly progression of the eucaryotic cell cycle and the cascade of intracellular signal transduction are two examples of processes driven and modulated by the phosphorylation state of key components. Among the many facets of protein function affected by phosphorylation are subcellular localization, participation in multiprotein complexes, enzymatic activity, and interaction with nucleic acids.

In this context, it is intriguing that vaccinia virus encodes two protein kinases and a protein phosphatase (2, 13, 17, 18, 25, 35). Vaccinia virus exhibits a high degree of physical and genetic autonomy from the host and encodes most, if not all, of the enzymes required for three temporally distinct phases of gene expression as well as DNA replication (20–22, 32–34). Moreover, it directs the morphogenesis of several classes of complex virions and encodes an extensive repertoire of proteins which interface with the immune and inflammatory responses of the host (27). There are thus numerous processes that might be regulated at the level of differential phosphorylation.

The H1 gene of vaccinia virus encodes a dual-specificity protein phosphatase (13), and we have recently shown that this enzyme plays a vital role in regulating the onset of early transcription (19). The B1 gene encodes an essential serine/threonine protein kinase; *ts* mutants with lesions in this gene are severely impaired in DNA replication (24, 25). Most recently, it has been reported that an additional protein kinase is encoded by the F10 gene (17). This kinase also shows specificity for serine and threonine residues; it is expressed at late times after infection and is encapsidated within vaccinia virions. The inability to isolate viable viral isolates lacking an intact F10 gene provides compelling evidence that the kinase plays an essential role in the viral life cycle.

One of the most fruitful approaches to functional analysis of a gene product is the phenotypic analysis of conditionally lethal mutants which bear lesions in the gene of interest. Two complementation groups of *ts* mutants from the collection of Condit and coworkers (5, 6, 31) have been shown to carry lesions that map within the right half of the 13.5-kb *Hin*dIII F fragment. These mutants, therefore, were potential candidates for elucidating the function of the F10 kinase.

The *ts* phenotypes of *ts*15, *ts*28, *ts*54 and *ts*61 are due to lesions within the F10 gene. *ts*30 and -48 and *ts*12, -15, -28, -54, -61 compose two complementation groups of temperature-sensitive (*ts*) mutants whose lesions have been localized to the right-hand half of the *Hin*dIII F fragment. We therefore investigated whether the F10 open reading frame (ORF) was capable of rescuing any of these mutants to the wild-type (wt) phenotype. Confluent monolayers of BSC40 cells (35-mm-diameter dishes) were infected at 32°C with the *ts* mutants at a multiplicity of infection (MOI) of 0.03. At 4 h postinfection (hpi), cultures were transfected with linearized plasmid DNA. Three plasmids were tested: (i) pBR322 containing the vaccinia virus genomic *Hin*dIII E fragment, (ii) pBR322 containing the vaccinia virus genomic *Hin*dIII F fragment, and (iii) pET14B containing the PCR-generated F10 ORF. DNA (3.3 μg) was applied to each dish as a calcium phosphate precipitate (8), and cultures were immediately shifted to 39.5°C. After 2 to 3 days of incubation, cells and medium were harvested and subjected to three cycles of freeze-thawing and sonication. Viral yield was determined by titration at 39.5°C. Neither *ts*30 nor *ts*48 was rescued by the F10 fragment, and therefore the lesions in these mutants must map to another ORF within *Hin*dIII-F (data not shown). In contrast, *ts*15, *ts*28, *ts*54 and *ts*61 were all rescued to the wt phenotype by the F10 ORF as well as by *Hin*dIII F (Table 1). Although *ts*12 was originally assigned to the same complementation group, it was not rescued to the wt phenotype by the F10 ORF or by the entire *Hin*dIII F fragment (data not shown). We therefore conclude that *ts*12 must have two lesions conferring *ts* phenotypes, one of which maps outside the *Hin*dIII F region. The data in Table 1 clearly show that the *ts* phenotypes of *ts*15, *ts*28, *ts*54, and *ts*61, on the other hand, are solely a function of lesions within the gene encoding the F10 protein kinase.

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TABLE 1. Marker rescue of *ts*28, *ts*15, *ts*54, and *ts*61

DNA fragment	PFU/ml[a]			
	*ts*28	*ts*15	*ts*54	*ts*61
*Hin*dIII E	$<10^2$	$<10^2$	$<10^2$	$<10^3$
*Hin*dIII F	2.9×10^6	2.5×10^5	5.0×10^4	2.2×10^5
F10	4.0×10^5	1.4×10^4	1.0×10^4	8.7×10^4

[a] Confluent monolayers of BSC40 cells were infected at 32°C with the indicated mutant at an MOI of 0.03. At 4 hpi, cultures were transfected with linearized preparations of plasmid DNA containing the indicated vaccinia virus genomic insert and shifted to 39.5°C. At 3 days postinfection, cultures were harvested and the viral yield was titrated at 39.5°C.

Phenotypic characterization of the F10 mutants. (i) Confirmation of a normal *ts* phenotype. The complementation group containing *ts*28, *ts*15, *ts*54, and *ts*61 was previously characterized as having a "normal" phenotype, i.e., the orderly progression through early, intermediate, and late gene expression was not perturbed at the nonpermissive temperature. By implication, DNA replication also proceeded normally. We have confirmed that viral protein synthesis proceeds normally during nonpermissive infections performed with *ts*28 (Fig. 1). However, plaque formation by all four mutants is compromised at the nonpermissive temperature (data not shown). *ts*28 and *ts*15 are highly restricted, forming no macroscopic plaques at 39.5°C, whereas *ts*54 and *ts*61 are far leakier, forming minute plaques at 39.5°C. This difference in the severities of the *ts* phenotype was confirmed by our comparison of viral yields obtained at 17 hpi from cultures infected at an MOI of 15 at either 32°C or 39.5°C (Fig. 2A). Relative to the yields obtained at 32°C, those obtained at 39.5°C were reduced by approximately 3, 2, 1, and 1 orders of magnitude for *ts*28, *ts*15, *ts*54, and *ts*61, respectively.

(ii) F10 mutants fail to form mature virions at the nonpermissive temperature. Normal mutants fall into two general categories, those that are defective in virion morphogenesis and those in which the virions produced are noninfectious in the next round of infection. To address this question, cultures were infected with *ts*28 at an MOI of 15 and maintained at 32 or 39.5°C for 24 h. Cells were then harvested, and virions were prepared from cytosolic lysates by sedimentation through 36% sucrose (43,000 × *g* for 80 min) and then through 25 to 40%

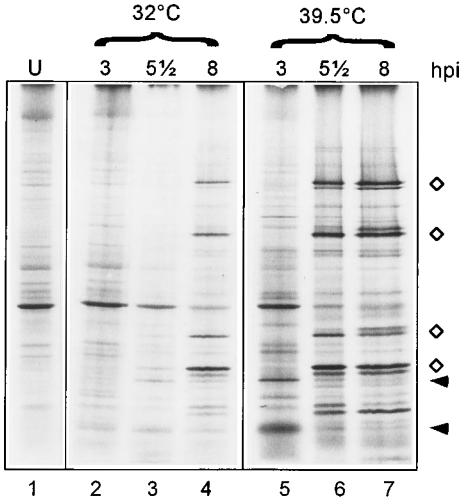

FIG. 1. *ts*28 displays a normal phenotype at the nonpermissive temperature. Confluent monolayers of BSC40 cells were infected at 32°C (lanes 2 to 4) or 39.5°C (lanes 5 to 7) with *ts*28 at an MOI of 15; at various times after infection, cultures were metabolically labeled with [^{35}S]methionine (100 μCi/ml) for 45 min and then harvested. The times indicated represent the time at harvest. Total cellular lysates were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Lane 1, extract prepared from uninfected (U) cells. Two representative early viral proteins, visible in lanes 2, 3, and 5 are indicated by arrowheads, and four characteristic late proteins, visible in lanes 4, 6, and 7, are indicated by diamonds.

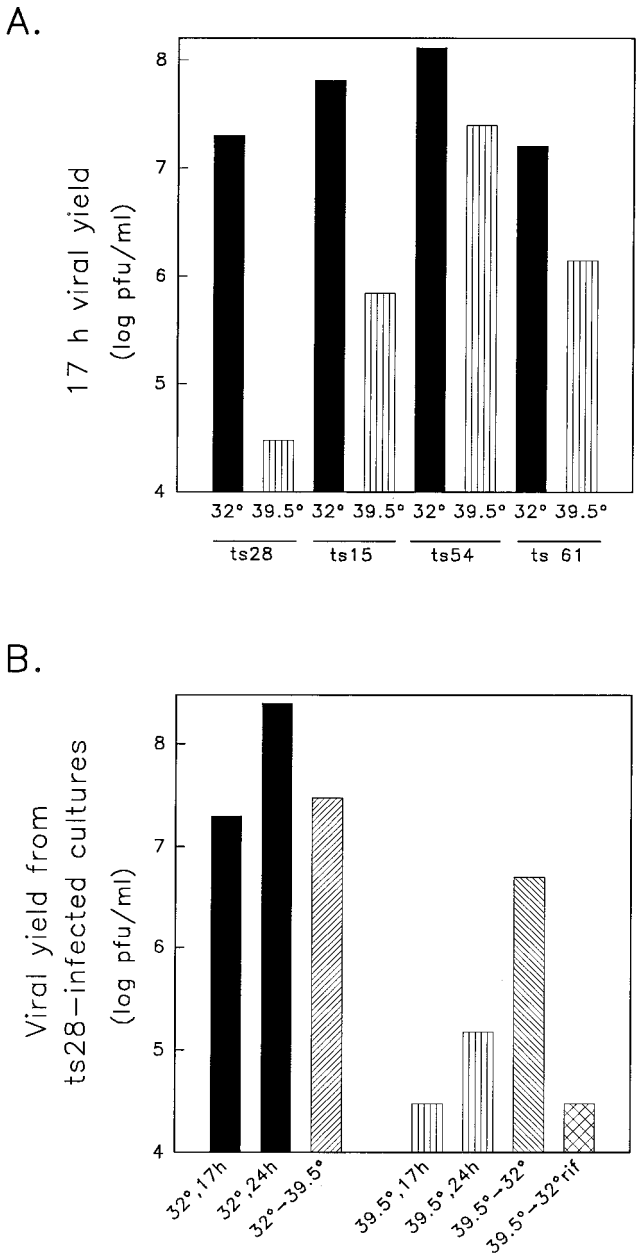

FIG. 2. Analysis of the viral yields produced during single cycles of infection with *ts*28, *ts*15, *ts*54, and *ts*61. (A) Confluent monolayers of BSC40 cells were infected with the virus shown at an MOI of 15 and maintained at either 32°C (solid bars) or 39.5°C (striped bars) for 17 h. Cells were then harvested, and cell-associated virus was titrated at 32°C. (B) Confluent monolayers of BSC40 cells were infected with *ts*28 at the temperature shown. Cultures were either maintained at 32°C (solid bars) or 39.5°C (striped bars) and harvested at 17 or 24 hpi or shifted from 32 to 39.5°C or from 39.5 to 32°C (hatched bars) at 17 hpi and then harvested at 24 hpi. Crosshatched bar, rifampin (rif) added to 100 μg/ml at the time of the temperature shift. For all samples, cell-associated virus was titrated at 32°C.

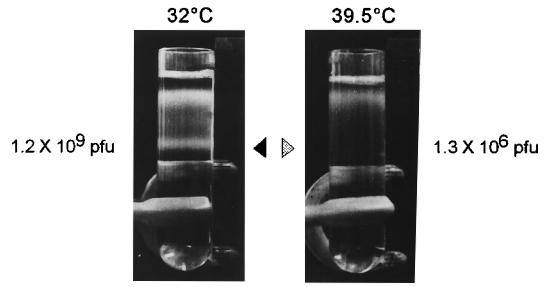

FIG. 3. Mature virions are not produced during nonpermissive infections with *ts*28. Confluent monolayers of BSC40 cells were infected with *ts*28 at an MOI of 15 and maintained at 32 or 39.5°C for 24 h. Cells were then harvested, and cell-associated virus was purified by ultracentrifugation as described in the text. Sucrose gradients after centrifugation are shown. Filled arrowhead, band of mature virions clearly visible in the sample harvested from cells infected at 32°C. No such band is present in the sample prepared from cells infected at 39.5°C (stippled arrowhead). The virion band was removed from the 32°C sample by needle aspiration, and an equal volume was also removed from the corresponding position in the 39.5°C sample. Virions were recovered by sedimentation and titrated at 32°C; the titers determined for each sample are shown.

sucrose gradients (6,000 × *g* for 45 min). It was immediately apparent that the characteristic band of virions seen in the 32°C preparations (filled arrowhead in Fig. 3) was absent in the 39.5°C preparations (stippled arrowhead in Fig. 3). The 32°C band was removed from the sucrose gradient by needle aspiration, concentrated by sedimentation (18,200 × *g* for 40 min), and titrated, as was an equal volume extracted from the same position in the banded 39.5°C preparation. The 32°C preparation contained 1,000-fold more infectious virus than did the 39.5°C preparation. These data confirm that cultures infected with *ts*28 at 39.5°C fail to produce mature virions.

(iii) The arrest in *ts*28-infected cultures is reversible and occurs prior to the rifampin-sensitive step. Further examination of the *ts* phenotype was performed with the mutant exhibiting the tightest temperature restriction, *ts*28 (Fig. 2B). Cultures arrested at the nonpermissive temperature for 17 h and then shifted to the permissive temperature for an additional 7 h (39.5°→32° in Fig. 2B) released a large burst of virus after the shift; these data are compatible with a reversible phenotype for *ts*28. Because these shifts were not performed in the presence of any pharmacological inhibitors such as cycloheximide, we cannot say whether de novo protein synthesis was required for this reversal or whether the F10 produced at 39.5°C regained activity upon shift to 31.5°C. Interestingly, this reversal was not seen when rifampin was added at the time of shift (39.5°→32°rif in Fig. 2B). Rifampin is known to block virion morphogenesis by inhibiting the association of the D13 protein with maturing membranes (1, 12, 29, 30, 37); in the presence of rifampin, flaccid membranous structures are formed, as are characteristic "inclusion bodies" of dense viroplasm. Were virion maturation in *ts*28-infected cultures to arrest at a point subsequent to the rifampin-sensitive stage, then one would expect a burst in virus production in cultures shifted to 31.5°C and simultaneously treated with the drug. That no such burst was seen strongly implies that the arrest in *ts*28-infected cultures occurs at a stage prior to the rifampin-sensitive step. These data are in agreement with our observation that, although late protein synthesis proceeded normally in *ts*28-infected cultures at 39.5°C (Fig. 1), proteolytic processing of the major structural proteins was not seen (data not shown) (16) and no mature virions were formed (Fig. 3).

Figure 2B also addresses the execution point of the *ts*28 mutation. Cultures maintained at 32°C for 17 h were shifted to 39.5°C for an additional 7 h of incubation (32°→39.5° in Fig. 2B); for comparison, parallel cultures were harvested at 17 hpi or left at 32°C until 24 hpi (solid bars in Fig. 2B). Essentially no additional infectious virus was produced after the cultures were shifted to 39.5°C, although cultures maintained at 32°C produced a significant amount of virus during this 7-h period. Synthesis of F10 protein at the permissive temperature until 17 hpi is apparently not sufficient to ensure ongoing morphogenesis of infectious virus after a temperature shift. These data argue against there being a pool of appropriately phosphorylated precursors en route to morphogenesis and suggest instead that F10 activity is required continuously for the assembly of infectious virions. Because this experiment measured infectious virus and not virion formation per se, it is possible that virion morphogenesis proceeds after the temperature shift but that the virions formed are not infectious. Such an interpretation implies that F10 would be required not only for virion formation but for the infectivity of assembled virions.

(iv) Nonpermissive infections with F10 mutants undergo arrest at the earliest stage of virion morphogenesis. To further address the apparent block to virion maturation in nonpermissive infections performed with *ts*28, we performed electron microscopic analysis of cultures infected with wt virus, *ts*28, *ts*15, *ts*54, or *ts*61 at both 32°C and 39.5°C (MOI 15). At 24 hpi the culture medium was removed by aspiration and cells were gently rinsed with phosphate-buffered saline (PBS) (140 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4 [pH 7.4]) and then fixed in situ with 1% glutaraldehyde in PBS for 15 min at room temperature. Cells were then harvested by scraping and collected by low-speed sedimentation. Cell pellets were then overlaid with 1% glutaraldehyde in PBS and incubated on ice for 1 h or overnight. Pellets were then postfixed in 1% osmium–1.5% potassium ferricyanide at room temperature for 1 h, washed extensively in 0.1 M sodium cacodylate, subjected to a graded series of dehydration steps, and embedded in LX112 resin (Ladd Research Ind., Burlington, Vt.). Samples were stained en bloc with 3% uranyl acetate in 50% ethanol; sections were poststained with 0.1% lead citrate. Grids containing thin sections were examined with a JEOL 100CX-II microscope.

In cultures infected with wt virus at either temperature, or in cultures infected with *ts* mutants at 32°C, all stages of virion morphogenesis were present. Crescents and immature particles, immature particles with nucleoids, and mature virions were readily seen (Fig. 4A and B). However, the profile was dramatically different in cultures infected with the *ts* mutants at 39.5°C. In cells infected with either *ts*28 or *ts*15, virtually no signs of virion morphogenesis were seen (Fig. 4C to F). Large areas of grainy viroplasm, devoid of cellular organelles, were the most striking feature of these infected cells. Some evidence of vesicular membranes was seen in these virosomes. In the vast majority of cells examined, no crescents, immature particles with or without nucleoids, or mature virions were seen. Thus, although late protein synthesis proceeds normally in these cultures, virion morphogenesis apparently arrests at the very earliest stage, i.e., prior to the formation of crescents.

The phenotype seen with *ts*54 and *ts*61 was somewhat different. Approximately 50% of the cells infected with *ts*61 at 39.5°C also exhibited expansive virosomes devoid of any evidence of virion maturation (Fig. 4G); however, crescents and immature virions without nucleoids were seen in the other 50% of the cells (Fig. 4H). Crescents and immature virions without nucleoids were seen in most cells infected with *ts*54 at 39.5°C (Fig. 4I), and occasional cells also contained mature virions. These observations are consistent with the observed leakiness of *ts*54 and *ts*61 at 39.5°C, as assessed both by 17-h viral yields (Fig. 2A) and by plaque assays.

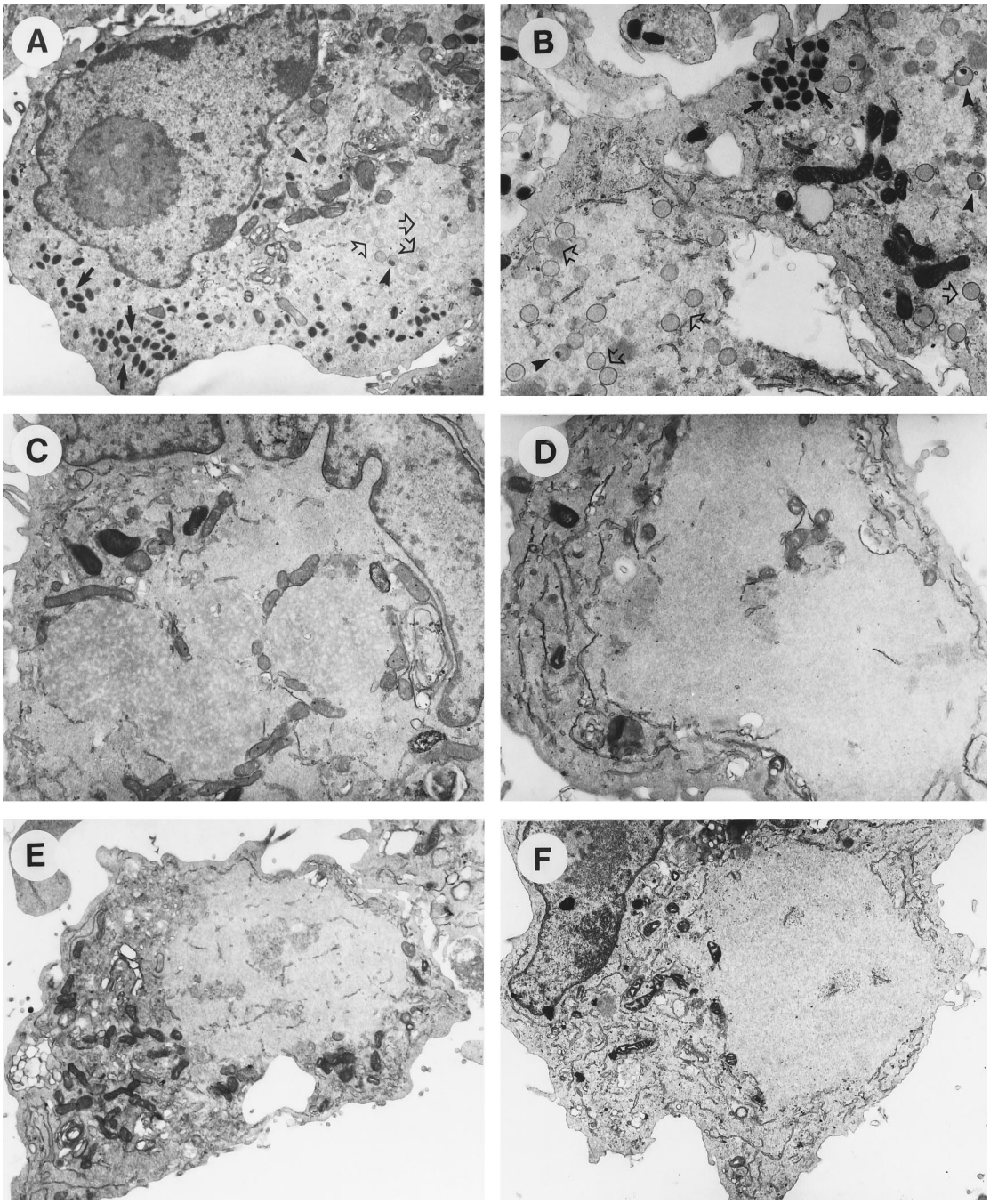

FIG. 4. Virion morphogenesis undergoes arrest at a very early stage in cells infected with *ts*28, *ts*15, *ts*61, and *ts*54 at the nonpermissive temperature. Confluent monolayers of BSC40 cells were infected with wt virus, *ts*28, *ts*15, *ts*61, or *ts*54 at an MOI of 15 and maintained at 32 or 39.5°C until 24 hpi. Cultures were fixed in situ and cells were then harvested and processed for transmission electron microscopy as described in the text. Representative fields were photographed. (A) wt virus at 39.5°C; (B) *ts*28 at 32°C; (C and D) *ts*28 at 39.5°C; (E and F) *ts*15 at 39.5°C; (G and H) *ts*61 at 39.5°C; (I) *ts*54 at 39.5°C; (J) *ts*28 at 39.5°C for 17 h and then 32°C for 7 h in the presence of rifampin at 100 μg/ml. Note that all stages of virion maturation, including crescents and immature virions (open arrows), immature virions with nucleoids (arrowheads), and mature intracellular virions (filled arrows) can be seen in panels A and B. Obvious clear areas of viroplasm devoid of any evidence of virion maturation can be seen in panels C to G. Clear areas of cytoplasm with some crescents and immature virions (open arrows) can be seen in panels H and I. Distinctive dense inclusion bodies (stars) within the clear areas of viroplasm can be seen in panel J. Magnification: ×5,900 (A, E, and F), ×8,200 (B to D, G, and I), or ×11,400 (H and J).

Our analysis of viral yields from single rounds of infection (Fig. 2) indicated that the arrest in *ts*28-infected cells occurred prior to the rifampin-sensitive stage of infection. We therefore examined cells infected with *ts*28 and maintained at 39.5°C for 17 h prior to a shift to 32°C in the presence of rifampin. No signs of normal virion maturation were seen in these cells, but dense inclusion bodies were seen (Fig. 4J). These did not appear to be membrane delimited and therefore appear more like the inclusion bodies described previously than like the irregular membrane-enclosed structures known as rifampin

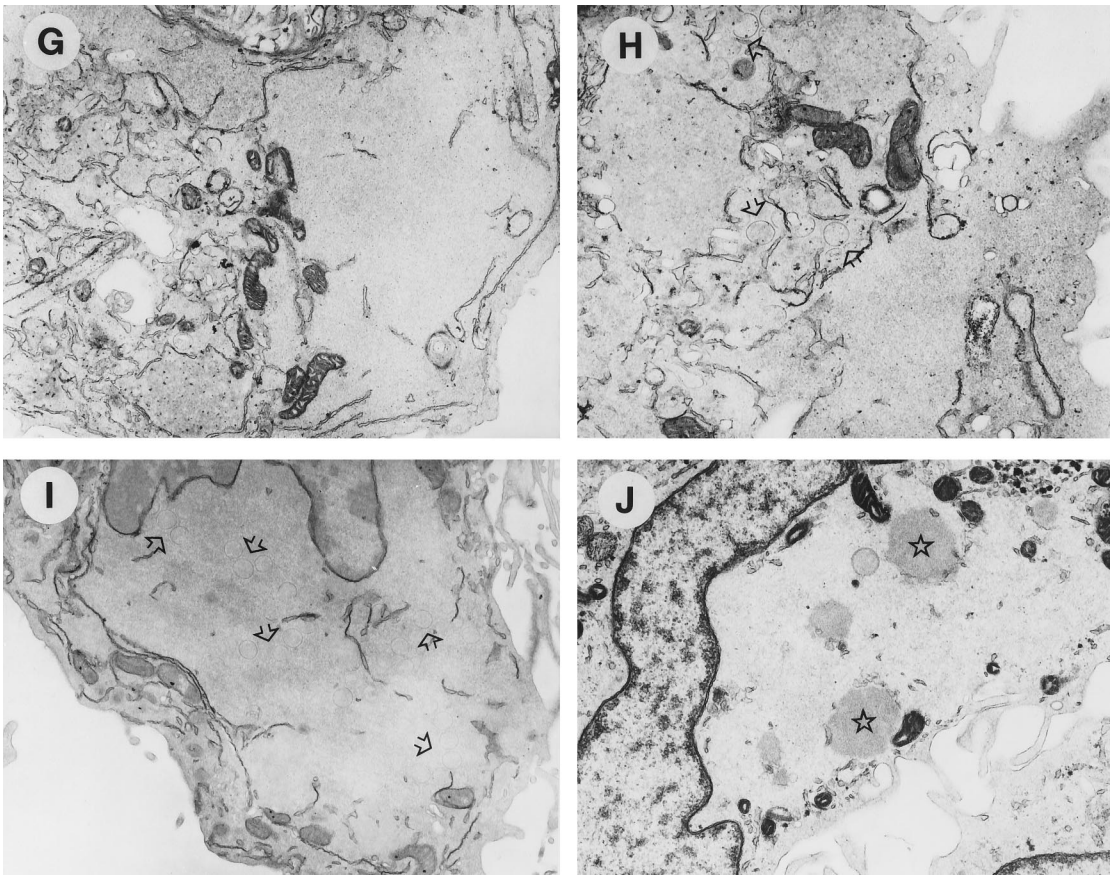

FIG. 4—*Continued.*

bodies (29, 37). Clearly, these data reinforce the conclusion that the arrest in *ts*28-infected cells occurs prior to the rifampin-sensitive step, prior to the earliest visible stages of virion morphogenesis.

This report shows definitively that the temperature-sensitive phenotypes of vaccinia virus mutants *ts*15, *ts*28, *ts*54, and *ts*61 are due to lesions in the F10 gene. Although plaque formation by these mutants is blocked at 39.5°C, these mutants display a normal phenotype in that synthesis of early and late viral proteins proceeds normally during a single infectious cycle at the nonpermissive temperature (Fig. 1) (5, 6, 31). Our studies show clearly that *ts*15, *ts*28, *ts*54, and *ts*61, however, fail to form mature virions at the nonpermissive temperature. Moreover, our electron microscopic analyses indicate that, in *ts*15 and *ts*28, this arrest of virion assembly occurs prior to the earliest visible signs of morphogenesis.

Our analyses indicate that the first defect seen during nonpermissive infections with F10 mutants occurs at the onset of morphogenesis. These data would seem to imply that the encapsidated F10 does not play a role during the early events of infection. However, a caveat to this interpretation is the observation by many other investigators that proteins synthesized and encapsidated at the permissive temperature are often stabilized and retain activity during the next round of (nonpermissive) infection. For example, *ts* mutants carrying lesions in the I8 helicase (9), the RNA polymerase (14), or the early transcription factor (vETF) (4) display a normal phenotype, although the encapsidated mutant proteins are clearly required during the early phase of infection. It thus cannot be firmly concluded that the F10 kinase is dispensable for stages of infection (early gene expression, uncoating, DNA replication, and intermediate or late gene expression) that precede virion morphogenesis. In an attempt to unmask such a role for F10, we have measured the infectivity of wt, *ts*15, *ts*28, *ts*54, and *ts*61 virions after preincubation at 45°C for 0 to 180 min. No significant or consistent increase in thermolability was observed for the *ts* virions (data not shown).

A variety of pharmacological agents and *ts* mutations arrest vaccinia virus maturation at various points (7). Dissection of the gene products involved in maturation, however, is still at a highly preliminary stage. The first sign of wt vaccinia virus morphogenesis occurs as membranes derived from the intermediate compartment (28), lying between the endoplasmic reticulum and the Golgi apparatus, are fashioned into crescents (7), which then develop into spherical immature virions. The presence of the D13 protein in the membranes of these crescents and immature virions is responsible for their characteristic shape and striated appearance (37). Nucleoid condensation then occurs, and an electron-dense, eccentric nucleoid appears within these immature virions. Proteolytic cleavages and morphological changes characterize the further progression to intracellular mature virions. A subset of these virions are destined for the extracellular environment, and they acquire an additional double envelope derived from the trans-Golgi network (26) and exit the cell in a process that involves fusion of the outermost lipid bilayer of the virion with the plasma membrane.

In the absence of the expression of the myristylated protein

L1, morphogenesis undergoes arrest after the formation of immature virions (23). Mutations in the F18 DNA-binding protein impair nucleoid condensation (36). Mutations in the I7 protein lead to aberrant immature virions whose interior has a characteristic half-moon appearance in which condensation of internal virion components is abnormal (15). Mutations in the D13 protein, a block to D13 expression, or the inclusion of the drug rifampin, which acts on the D13 protein, causes an earlier block to morphogenesis (37). Flaccid membrane-bound enclosures form; these enclosures lack the distinct shape and striated appearance of crescents and immature virions.

The studies reported here, involving *ts* mutants with lesions in the F10 protein kinase, describe the earliest morphogenetic arrest ascribed to a specific gene product to date. Nonpermissive infections undergo arrest prior to the formation of any visible signs of morphogenesis. Previously, this absence of maturation has been seen only with some mutants defective in late gene expression (14). In cultures infected nonpermissively with the most stringently *ts* mutants, *ts*15 and *ts*28, no crescents or immature particles were seen. The most striking features within these infected cells were the clear areas devoid of cellular organelles and characterized by a grainy appearance. These areas are presumably the cytoplasmic sites in which vigorous DNA replication occurs and viral gene products accumulate. Because late viral gene expression is not impaired in the mutants carrying F10 lesions but virion morphogenesis is essentially absent, it appears that F10 and/or its associated kinase activity play a crucial role in the initiation of virion assembly.

It seems plausible that phosphorylation of a key substrate initiates the extension of intermediate-compartment membranes into crescents. Identification of the authentic substrates of F10 is thus of clear interest. In this regard, it is provocative that we have found that the H5 protein is a good substrate for F10-mediated phosphorylation in vitro (data not shown). The H5 protein (3, 10, 11) is an abundant phosphoprotein which is a major component of virosomes and of the membranes of intracellular mature virions; antisera to H5 can neutralize virion infectivity. Immunogold electron microscopy has revealed that H5 is associated with crescents and immature virions, and this association appears to persist even when the D13 protein is mutated and hence absent from nascent virion membranes. Thus, association of H5 with maturing membranes occurs at the earliest stages of morphogenesis, and therefore the demonstration that H5 is a substrate for F10 is indeed provocative.

When *ts*28-infected cultures maintained at 32°C for 17 h were shifted to 39.5°C for an additional 7 h, no further infectious virus was produced. These data imply that functional F10 is required for ongoing virion assembly and, by extension, that there is not a pool of appropriately phosphorylated precursors in line for encapsidation. Rather, we propose that F10 may act stoichiometrically to initiate assembly, perhaps by forming a ternary complex with a membrane-bound substrate which leads to crescent extension with concomitant encapsidation of F10 itself. The specific defect in each of the *ts* alleles remains to be discovered; at this time we cannot determine whether kinase activity, protein stability, or substrate recognition are impaired in the various mutants.

A caveat to genetic analysis is the limitation that it is the first lethal phenotype that is seen and that this lethality masks any role the impaired gene product may play in later events. Infection with the F10 mutants undergoes arrest prior to the appearance of any recognizable components of assembling virions. Nevertheless, it may well be that F10 also plays a role later in virion assembly and that the encapsidation of F10 reflects this role. For example, the complex transition from immature to mature virions remains poorly understood; our only clue is that proteolytic processing accompanies dramatic morphological reorganization. It is tempting to speculate that encapsidated F10 may mediate phosphorylation events that also participate in driving the progression of virion assembly.

Protein phosphorylation unquestionably plays a major role in regulating various cellular processes such as progression through the cell cycle and transduction of extracellular signals to the nucleus. Phosphorylation affects protein-protein interactions and subcellular compartmentalization, as well as protein-nucleic acid interactions. In this regard, it is intriguing that the F10 kinase may well play the key role in orchestrating the protein-protein and protein-membrane interactions that precipitate virion morphogenesis. By analogy, one might predict that similar kinase-substrate interactions will be shown to propel the assembly of cellular organelles.

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