JOURNAL OF VIROLOGY, Feb. 1989, p. 714–729
0022-538X/89/020714-16$02.00/0

Vol. 63, No. 2

Detailed Phenotypic Characterization of Five Temperature-Sensitive Mutants in the 22- and 147-Kilodalton Subunits of Vaccinia Virus DNA-Dependent RNA Polymerase

URVASHI HOODA-DHINGRA, CAROL L. THOMPSON, AND RICHARD C. CONDIT*

Department of Biochemistry, State University of New York at Buffalo, 140 Farber Hall, Buffalo, New York 14214

Received 19 August 1988/Accepted 30 October 1988

We have carried out detailed phenotypic characterization of five temperature-sensitive (*ts*) mutants of vaccinia virus, the *ts* lesions of which have previously been mapped to two different subunits of the viral RNA polymerase. We have also attempted to determine the mechanism of temperature sensitivity in these mutants. Phenotypic characterization of each of the mutants showed that at the nonpermissive temperature, all five mutants exhibited normal levels of early viral mRNA and protein synthesis, but for an extended period of time, all mutants accumulated normal levels of DNA in abnormally large pools in the cell cytoplasm; all mutants were defective in the synthesis of late viral mRNA and proteins and in viral morphogenesis. In an attempt to address the mechanism of temperature sensitivity in these mutants, we measured the effect of a temperature shift on the ability of the mutants to direct late viral protein synthesis. If infected cells were shifted down from a nonpermissive temperature late during infection, late protein synthesis was initiated after a lag period of 1 to 2 h. If infected cells were shifted up from a permissive temperature early during infection, late protein synthesis continued to be defective. If infected cells were shifted up to the nonpermissive temperature after late protein synthesis had commenced, late protein synthesis was maintained at the nonpermissive temperature at the level observed when the temperature was shifted up. We interpret these results to mean that once a functional RNA polymerase has been assembled at the permissive temperature during a mutant infection, it remains functional at the nonpermissive temperature, but that the *ts* mutants are defective in the assembly of a newly synthesized RNA polymerase at the nonpermissive temperature. This interpretation implies that the virion RNA polymerase is responsible for early viral transcription and that a newly synthesized RNA polymerase transcribes late viral genes.

Vaccinia virus, a large double-stranded DNA-containing virus, is distinguished by its unusual morphology and cytoplasmic site of replication. Like many other DNA viruses, its gene expression is temporally regulated but it is unique in that its gene expression is carried out by virus-encoded, host-independent transcriptional machinery (27). Upon entry into the host cell, the virion-associated enzymes transcribe early viral genes until viral DNA replication begins. When DNA replication begins, expression of most early genes ceases and late viral gene expression is initiated. The mechanism controlling the switch from early to late viral gene expression is poorly understood. If conditional lethal mutations which affect the switch could be isolated, then the characterization of the mutants would aid in the identification of factors controlling the switch from early to late viral gene expression.

We have previously isolated 65 temperature-sensitive (*ts*) mutants of vaccinia virus, assigned the mutants to complementation groups, and carried out a preliminary characterization of the mutants (7, 8). Analysis of the data revealed that mutants in six different complementation groups were defective in switching from early to late viral gene expression (8). We have identified two of these complementation groups as genes encoding two different subunits of the viral RNA polymerase (presented in an accompanying report [43]). This suggested that the RNA polymerase might be one of the factors involved in the switch from early to late gene expression. We therefore have undertaken a detailed phenotypic characterization of these mutants and attempted to identify the mechanism of temperature sensitivity in the mutants.

There are pronounced differences in the structure of early and late vaccinia virus mRNAs which could relate to the switch from early to late gene expression. Early mRNAs are of a discrete size with homogeneous 5′ and 3′ ends, a result of at least two different *cis*-acting elements which control initiation (6, 9, 44) and termination (33, 47) of early transcription. The 5′-flanking regions from early genes behave like classical promoters in a variety of in vitro and in vivo assays. In contrast, late mRNAs are heterogeneous at both the 5′ and 3′ ends. Many, if not all, late mRNAs have a poly(A) sequence of 30 to 50 residues, which is not encoded in the genome, attached to their 5′ ends (3, 35, 36). Some late genes produce mRNAs with multiple 5′ ends, resulting from transcription of varying amounts of 5′-flanking sequence (19, 20). Specific termination of transcription does not occur late during infection, so late transcripts are also heterogeneous at their 3′ ends (22). Although the 5′-flanking regions of late genes are clearly essential in conferring late transcription specificity to these genes (2, 6, 17, 25, 45), the relationship of these sequences to poly(A) heads or to a typical promoter sequence is not yet understood.

Differences in the regulatory sequences between early and late viral genes also suggest that the transcription complexes that interact with these sequences may differ. Core RNA polymerase purified from virions is a heteropolymer (1, 41) with immunologic cross-reactivity (26) and amino acid sequence homology (4) with cellular RNA polymerase II. The core enzyme has only nonspecific polymerizing activity in vitro (1, 41). However, a high-molecular-weight protein

* Corresponding author.

complex which is capable of accurate transcription initiation and termination of vaccinia virus early genes in vitro can be isolated from virions (5). This suggests that additional factors interact with the core polymerase to render it specific. Although a complete characterization of the virion-associated transcription complex is not yet available, it has been shown that the viral guanylyl transferase and a viral DNA-dependent ATPase cosediment with the complex along with other polypeptides of unknown function (5). Further fractionation of the transcription complex has led to purification of a viral transcription termination factor which copurifies with the viral guanylyl transferase (39). An in vitro system which is derived from vaccinia virus-infected cells and which accurately transcribes late genes has recently been reported (35). There is as yet insufficient data to permit a detailed comparison of this system with the virion-associated complex. However, the inability of the virion-associated transcription complex to transcribe late genes and the differences in early and late gene regulatory sequences and in the structures of the mRNA products all suggest that the transcription system specific for late genes differ from the virion-associated transcription complex and that this difference is a critical determinant in the switch from early to late viral gene expression.

It has been suggested that the viral RNA polymerase core enzyme may play an integral role in the switch from early to late gene expression in vaccinia virus (14). Phenotypic characterization of *ts* mutants with lesions in the 147-kilodalton (kDa) subunit of the RNA polymerase led Ensinger (14) to suggest that newly synthesized RNA polymerase may be responsible for late transcription or that the virion-associated RNA polymerase may interact with factors to discriminate between early and late promoters.

We report here the phenotypic characterization of five *ts* mutants, the *ts* lesions of which have been mapped to either the 22- or 147-kDa subunit of the viral RNA polymerase. At the nonpermissive temperature, all the mutants show normal levels of early viral mRNA and synthesize normal amounts of early viral proteins, but for an extended period of time, all mutants accumulate normal amounts of viral DNA in abnormally large pools in the cell cytoplasm and all are defective in the synthesis of late viral mRNA and proteins. In an attempt to identify the molecular defect in these mutants, we studied the effect of temperature shifts on protein synthesis patterns of the mutants and the results are presented in this report. We interpret the data obtained to mean that the mutants are defective in the assembly of RNA polymerase subunits into a functional complex at the nonpermissive temperature. These results imply that the virion-associated RNA polymerase transcribes early vaccinia virus genes and that a newly synthesized RNA polymerase transcribes late genes.

MATERIALS AND METHODS

Cells and virus. BSC40 cells, wild-type (wt) vaccinia virus strain WR, *ts*7, *ts*20, *ts*42, *ts*51, *ts*53, *ts*65, the conditions for their growth, infections, and plaque titrations have been described in detail previously (7, 8).

Bacteriophage clones. The construction of the recombinant phages RI3300.044 and Bm3205.202, the map positions of their inserts, and the conditions for their growth have been described in detail previously (21).

Protein synthesis. Pulse labeling of infected cells with [^{35}S]methionine (New England Nuclear Corp.), sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and autoradiography procedures have been previously described (7).

DNA synthesis. Measurement of viral DNA replication, using dot blot hybridization, was done as described previously (14), with the modifications described below. Cells in 35-mm-diameter dishes were infected with 20 PFU per cell of mutant or wt virus, and the dishes containing the infected cells were incubated at 31 or 40°C. GeneScreen was used as the hybridization membrane. Hybridization and washes were done as described in the GeneScreen instruction manual. For a probe, plasmid clone 801 containing a 2,130-base-pair *Eco*RI-*Sal*I fragment from open reading frame D7R (28) was cut with the restriction enzymes *Eco*RI and *Hpa*I. A 1,600-base-pair *Eco*RI-*Hpa*I DNA fragment was purified by gel electrophoresis and labeled with [^{32}P]dCTP (ICN Pharmaceuticals Inc.) by nick translation. The unincorporated nucleotides were removed by using a spun column (23).

Isolation of total RNA from infected cells. Cells in individual (100-mm-diameter) dishes were infected with 20 PFU per cell of either wt or mutant virus and incubated at 31 and 40°C as described above. At various times postinfection, total RNA was isolated from the infected cells as described previously (31).

Northern (RNA) analysis. Formaldehyde-treated RNA (38), isolated from virus-infected cells, was fractionated by electrophoresis on a 1.2% agarose gel and then transferred to GeneScreen. The gels were stained with ethidium bromide (3 μg/ml in electrophoresis buffer) before and after transfer to the membrane. After the RNA was transferred, the membrane was rinsed in 12.5 mM sodium phosphate buffer, dried, and then baked at 80°C for 3 h. Prehybridizations and hybridizations were done as described in the GeneScreen instruction manual. The radioactive probe used in the hybridization was made as described previously (21) with the modifications described below. A [^{32}P]DNA copy of single-stranded M13mp18 DNA with a specific activity of 5×10^8 cpm per μg of DNA was made by the oligolabeling procedure provided with the oligolabeling kit (Pharmacia, Inc.). The unincorporated nucleotides were removed by using a spun column (23). Two micrograms of the unlabeled single-stranded recombinant phage RI3300.044 or Bm3205.202 (21) DNA was mixed with 20 ng of the [^{32}P]DNA copy of M13, boiled for 10 min, and allowed to anneal for 6 to 10 h at 55°C. The annealed probe containing 1×10^8 to 2×10^8 cpm was then added to the hybridization mixture, and the hybridization was continued for 16 to 24 h at 42°C. The filters were washed twice for 5 min each at room temperature with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and then three times for 30 min each at 65°C with 0.1× SSC–1% SDS. The filters were then dried and autoradiographed by using Kodak XAR-5 film and an intensifying screen (Cronex Lightning-Plus, E. I. du Pont de Nemours & Co., Inc.) at −70°C.

Temperature shifts and effect of ActD. Cells in individual 35-mm-diameter dishes were infected with 20 PFU of mutant virus per cell at 31 and 40°C for 30 min. The inoculum was removed, the dishes were washed twice with prewarmed phosphate-buffered saline, fresh medium was added, and the dishes were incubated at 31 and 40°C. At 4, 6, and 8 h postinfection, sets of dishes were transferred from 31 to 40°C and from 40 to 31°C without changing the medium on the cells. Medium on cells in other sets of dishes was replaced with medium containing 10 μg of actinomycin D (ActD) per ml, and the dishes were transferred from 31 to 40°C and from 40 to 31°C. At various times, the infected cells were pulse-labeled with [^{35}S]methionine for 15 min. Protein samples

were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Electron microscopy. BSC40 cells grown to confluence on cover slips coated with coverslip release spray (Miller-Stevenson) were infected with virus at a multiplicity of infection of 20 PFU per cell. After a 0.5-h adsorption period at the appropriate temperature, excess inoculum was washed from the monolayers, medium was added, and infected cells were incubated at 31 or 40°C for the indicated times. Infected cells were fixed as described previously (24), and embedded in the resin described by Spurr (42). Sections were made in the plane of the monolayer and processed for electron microscopy by conventional techniques. Samples were examined by using a JEOL 100 CX-2 transmission electron microscope.

Feulgen staining. Cells were grown on cover slips and infected as described for electron microscopy. Cells were then rinsed with phosphate-buffered saline, fixed with neutral buffered 10% Formalin, and stained by conventional techniques (32).

RESULTS

We have previously identified three *ts* mutants, *ts*51, *ts*53, and *ts*65, and two other *ts* mutants, *ts*7 and *ts*20, that have lesions in the 147- and 22-kDa subunits of the vaccinia virus RNA polymerase, respectively (presented in the accompanying report [43]). Preliminary characterization of these five *ts* mutants has also been previously reported (7, 8). Data from those studies indicated that, at the nonpermissive temperature, all five *ts* mutants synthesized normal amounts of early viral proteins. Two of the mutants, *ts*51 and *ts*65, appeared to shut off early protein synthesis normally and synthesize normal amounts of late viral proteins at the nonpermissive temperature. However, the other three mutants, *ts*53, *ts*7, and *ts*20, did not shut off early viral protein synthesis and synthesized subnormal amounts of late proteins. These preliminary observations indicated that most of the mutants with lesions in RNA polymerase subunits have a functional early transcription apparatus but are defective in turning off early viral gene expression and turning on late viral gene expression. These results suggested a functional role for the viral RNA polymerase in the switch from early to late gene expression in vaccinia virus. We therefore decided to carry out a detailed phenotypic characterization of these mutants to identify the molecular defect in the viral RNA polymerase.

Protein synthesis in virus-infected cells. We reexamined and quantitated viral protein synthesis in cells infected with each of the five mutants. Infected cells were pulse-labeled with [^{35}S]methionine at various times postinfection at 31 and 40°C. The labeled polypeptides were separated on an SDS-polyacrylamide gel and visualized by autoradiography. Autoradiograms of protein synthesis in cells infected with wt, *ts*7, *ts*20, *ts*51, *ts*53, and *ts*65 are shown in Fig. 1. An early and a late protein (shown by an arrow in Fig. 1) were chosen as representatives of early and late viral protein synthesis for densitometric analysis. A graphical representation of the densitometric analysis of these two proteins in the wt- and mutant-infected cells is shown in Fig. 2. The data indicated the following. (i) The infection by wt virus was hastened at 40°C compared with that at 31°C (Fig. 1a). Specifically, early viral protein synthesis and host protein synthesis were shut off earlier at 40°C (Fig. 1a and 2a). Late protein synthesis commenced earlier at 40°C and the amount of late proteins synthesized was higher at 40°C (Fig. 1a and 2b). (ii) In cells infected with *ts*7, *ts*20, *ts*53, or *ts*65, early and late viral proteins were synthesized in a normal fashion at 31°C, whereas very little, if any, late viral protein synthesis was observed at 40°C (Fig. 1b, c, e, and f and 2a and b). In addition, the shutoff of host protein synthesis at 40°C after infection with these mutant viruses was apparently unaffected, whereas early virus proteins were synthesized for a prolonged period of time (Fig. 1b, c, e, and f, and 2a). (iii) In cells infected with *ts*51, early viral proteins were synthesized in normal amounts at 31 and 40°C (Fig. 1d and 2a). Late proteins were synthesized in the same amounts at 31 and 40°C but synthesis was delayed by 2 to 4 h at 40°C (Fig. 1d and 2b). The shutoff of host and early viral protein synthesis in *ts*51-infected cells at 40°C was normal (Fig. 1d and 2a). In summary, these data show that *ts*51 was slightly defective in the synthesis of late viral proteins whereas *ts*7, *ts*20, *ts*53, and *ts*65 were strongly defective in turning off early viral protein synthesis and turning on late viral protein synthesis.

Northern analysis. The results described above show that *ts*7, *ts*20, *ts*53, and *ts*65 are defective in switching from early to late viral protein synthesis. Since these mutants possess *ts* lesions in RNA polymerase subunits, it is highly probable that the observed defect in the protein synthesis patterns reflects a defect at transcriptional, rather than translational, level. We therefore assayed, by Northern analysis, the steady-state levels of early and late viral mRNA in wt- and mutant virus-infected cells. We chose one mutant each, *ts*7 and *ts*53, from the 22- and 147-kDa viral RNA polymerase subunits, respectively. We determined the levels of mRNA transcribed from one early (D5R) and one late (D13L) gene in the *Hin*dIII D fragment as overall representatives of early and late viral gene expression. Total RNA, isolated from infected cells at various times postinfection was separated on an agarose gel and transferred to GeneScreen. The filters were probed with single-stranded recombinant phage RI3300.044 or Bm3205.202, both of which have been shown to specifically hybridize to transcripts from either gene D5R or gene D13L, respectively (21). The autoradiograms are shown in Fig. 3. The data showed that, in wt-infected cells, a transcript encoding the early gene D5R was observed only at 2, 4, and 6 h postinfection at 31°C and in higher amounts at 2 and 4 h postinfection at 40°C. The smear in the background observed at late times during the infection is characteristic of a late transcript, probably D3R, which is transcribed in the same direction as gene D5R and reads through gene D3R (Fig. 3a). It is therefore detected by the single-stranded probe specific for gene D5R. In *ts*7-infected cells, at 31°C, the transcript encoding the early gene D5R was present in the same amount and at the same times postinfection as in the wt-infected cells. In contrast to wt-infected cells at 40°C, the D5R transcript persisted until 12 h postinfection in mutant-infected cells (Fig. 3a). Similar results were obtained with *ts*53-infected cells (data not shown). When the wt-infected cells were probed for the D13L late message, a heterogeneous smear characteristic of late vaccinia virus transcripts was observed after 6 h postinfection at 31°C and at 4 h postinfection at 40°C, consistent with the hastened late protein synthesis patterns observed at 40°C (Fig. 3b). In sharp contrast, in *ts*7- and *ts*53-infected cells, the steady-state levels of D13 message were severely reduced at 40°C, consistent with the subnormal late protein synthesis pattern exhibited by the mutants at 40°C (Fig. 3b). On the basis of these results, we conclude that the mutants *ts*7 and *ts*53 are unable to turn off the transcription of early viral genes and turn on the transcription of late viral genes normally at the nonpermissive temperature.

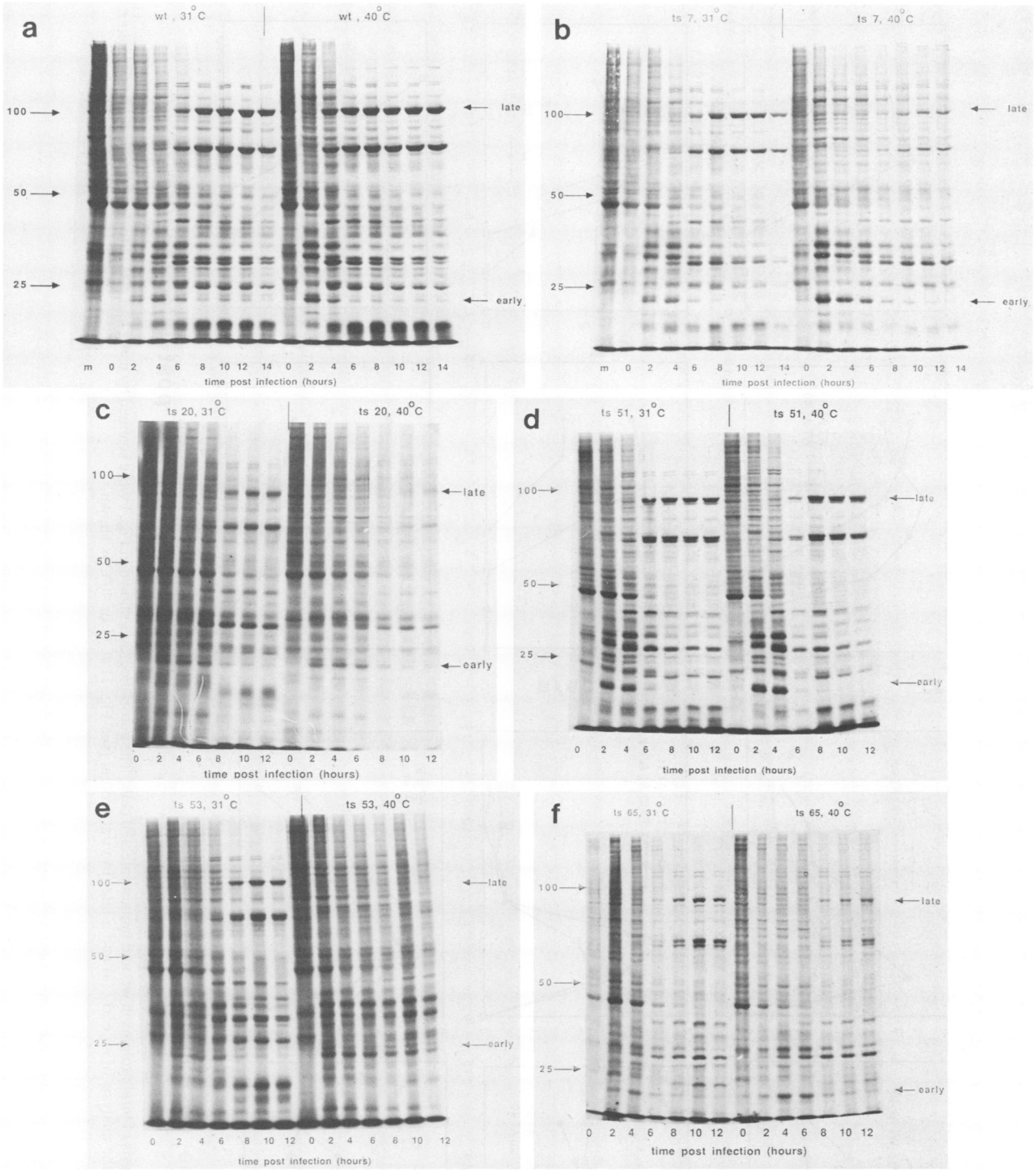


FIG. 1. Protein synthesis of wt- and mutant-infected cells. Cells were infected at 31 or 40°C with the virus indicated at the top of each figure and then pulse-labeled with [^{35}S]methionine. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and autoradiographed. The autoradiograms are shown. Each lane represents pulse-labeling at a different time postinfection shown at the bottom of each autoradiogram. Approximate molecular masses (in kilodaltons) are indicated to the left. On the right are indicated an early and a late protein which were used for densitometric analysis. Lanes m, Mock-infected cells.

Viral DNA synthesis. The data from protein synthesis experiments and Northern analysis indicated that four of the five mutants in the RNA polymerase subunits were unable to turn off early viral transcription and turn on late viral transcription. A plausible explanation for the above observations is that the mutants are defective in DNA replication, since previous studies have indicated that mutants defective in DNA replication exhibited a similar phenotype (8). To

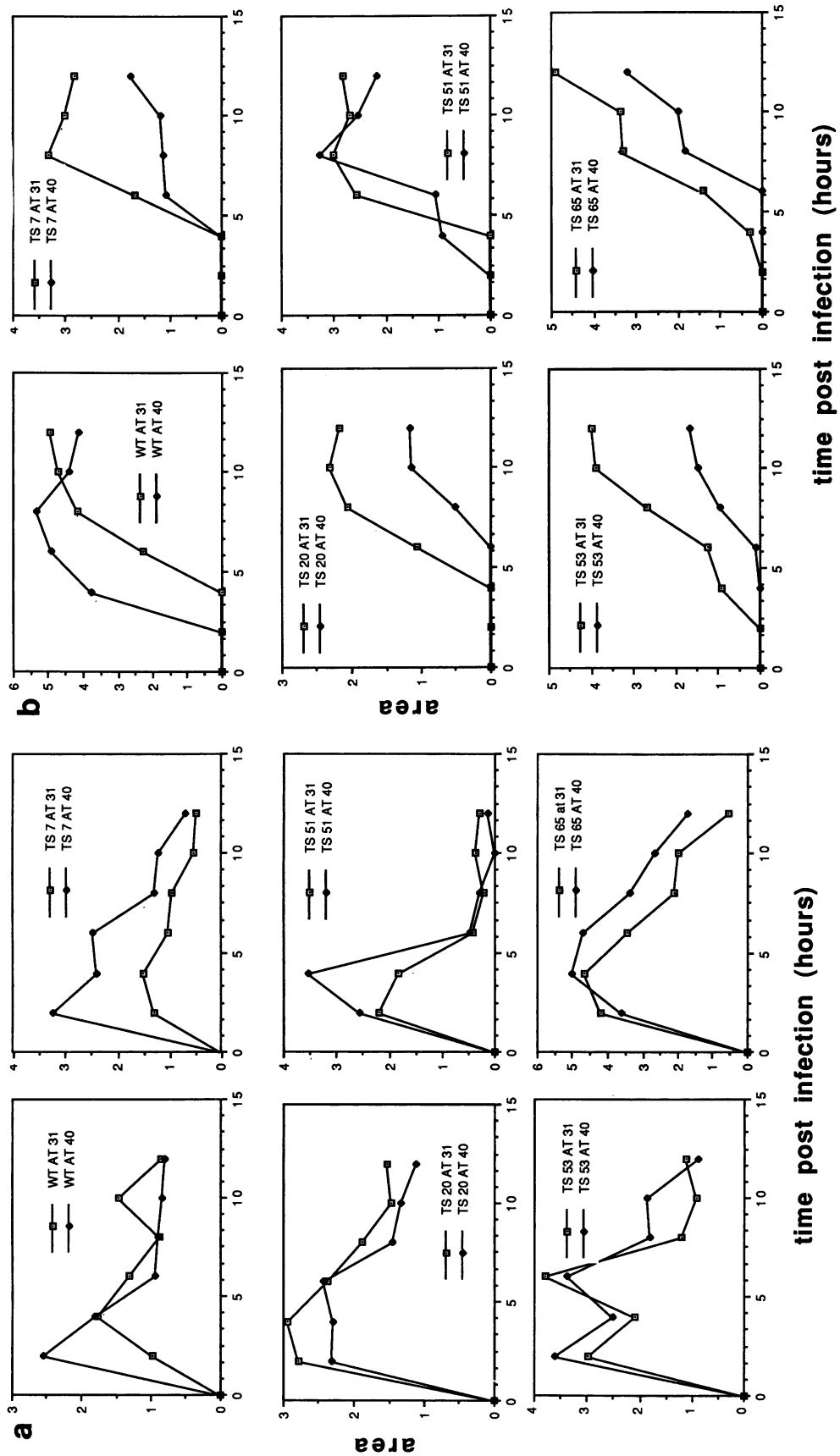


FIG. 2. Densitometric analysis of viral proteins in wt- and mutant-infected cells. The autoradiograms shown in Fig. 1 were scanned by using an LKB UltroScan XL laser densitometer. A graphical representation of the analysis of an early (a) and a late (b) viral protein (see Fig. 1) at 31 or 40°C is shown.

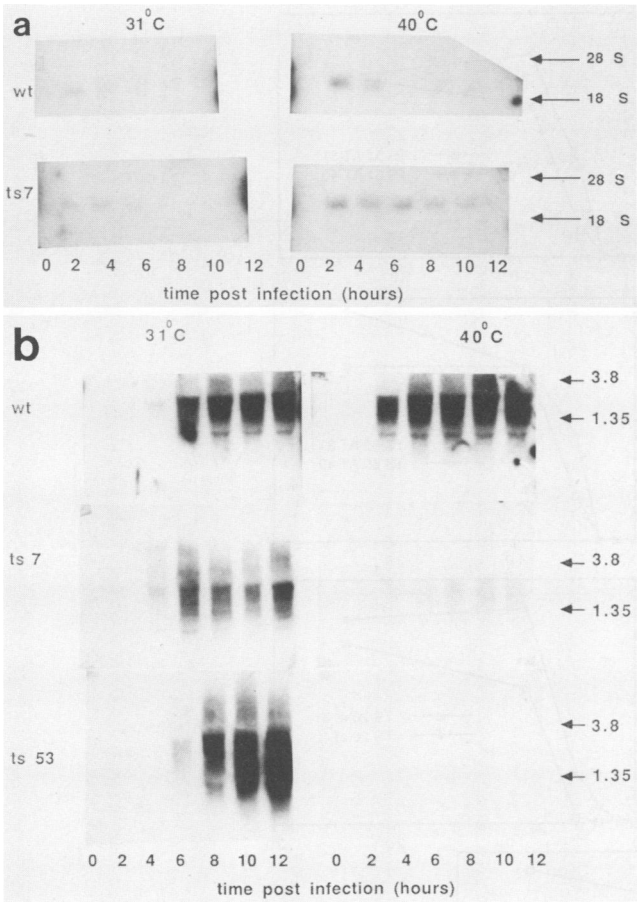


FIG. 3. Northern analysis of infected-cell RNA. BSC40 cells were infected with wt, *ts*7, or *ts*53 and incubated at 31 or 40°C. Total RNA was extracted at various times postinfection, indicated at the bottom of the figure, and blotted onto GeneScreen. The filters were probed with ^{32}P-labeled M13 clones that specifically hybridize to transcripts from gene D5 (a) or gene D13 (b) (19). The filters were then washed and autoradiographed (see Materials and Methods). The autoradiograms are shown. (a) The positions to which the 18S and 28S rRNA migrate are indicated on the right. (b) The positions to which other labeled markers migrate are indicated on the right.

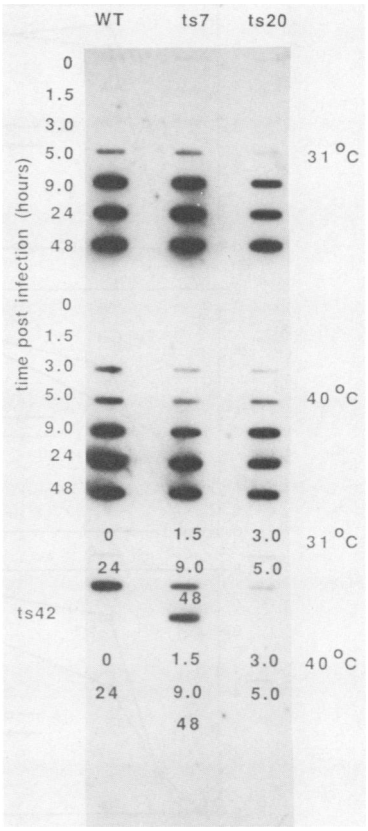


FIG. 4. Dot blot analysis of virus-specific DNA accumulation in wt- and mutant-infected cells. BSC40 cells were infected with wt (WT) or mutant virus at 31 or 40°C. At the time indicated (shown to the left in the top half of the autoradiogram and on the autoradiogram itself in the bottom half) postinfection, crude extracts of the virus-infected cells were blotted onto GeneScreen, probed with a ^{32}P-labeled virus-specific DNA fragment, washed, and autoradiographed (see Materials and Methods). The autoradiogram is shown.

address this possibility, we measured the accumulation of virus-specific DNA sequences in cells infected with the mutants. Crude extracts of virus-infected cells made at various times postinfection were blotted onto GeneScreen, denatured, probed with a recombinant viral DNA fragment, and autoradiographed. The autoradiogram depicting the pattern of DNA accumulation in wt-, *ts*7-, and *ts*20-infected cells is shown in Fig. 4. *ts*42, a DNA-negative mutant (a mutant that does not synthesize DNA at the nonpermissive temperature) (8), served as a negative control in this experiment. A similar experiment was done with *ts*51-, *ts*53-, and *ts*65-infected cells (data not shown). Densitometric analysis of the results obtained from all six mutants is shown in Fig. 5. The data showed that viral DNA accumulation in wt-infected cells was first observed at 5 h postinfection at 31°C and at 3 h postinfection at 40°C. In the DNA-negative mutant *ts*42, viral DNA accumulation was first observed at 5 h postinfection at 31°C and no DNA accumulation was observed at 40°C. All five *ts* mutants showed normal accumulation of viral DNA at 40°C compared with that of wt virus.

Electron microscopy. The poxvirus infection is particularly amenable to electron microscopic analysis, and detailed descriptions of the development of both wt (10, 12) and mutant (11, 13, 15, 40) vaccinia virus strains have been published. Normal morphogenesis begins with the appearance of characteristically rigid viral membranes in cytoplasmic areas of viral replication termed viroplasm. These membranes eventually close to form nucleoprotein-containing vesicles called immature particles. The structure of the immature particles is then reorganized to yield mature particles which contain a biconcave, DNA-containing core flanked by lateral bodies, surrounded by the viral membrane. In very few circumstances has an electron microscopic phenotype been correlated with a precise biochemical phenotype for a conditional lethal mutant of the virus (13, 15, 40). We were therefore interested to examine, by electron microscopy, the appearance of cells infected with RNA polymerase mutants.

Cells grown on cover slips were infected with wt or mutant virus, incubated at 31 or 40°C for various periods of time, fixed, embedded, sectioned in the plane of the monolayer, stained, and examined in the electron microscope. Typical sections are shown in Fig. 6. In cells infected with wt virus for 24 h at 40°C (Fig. 6b), viroplasm can be discerned as regions containing a density of mitochondria lower than

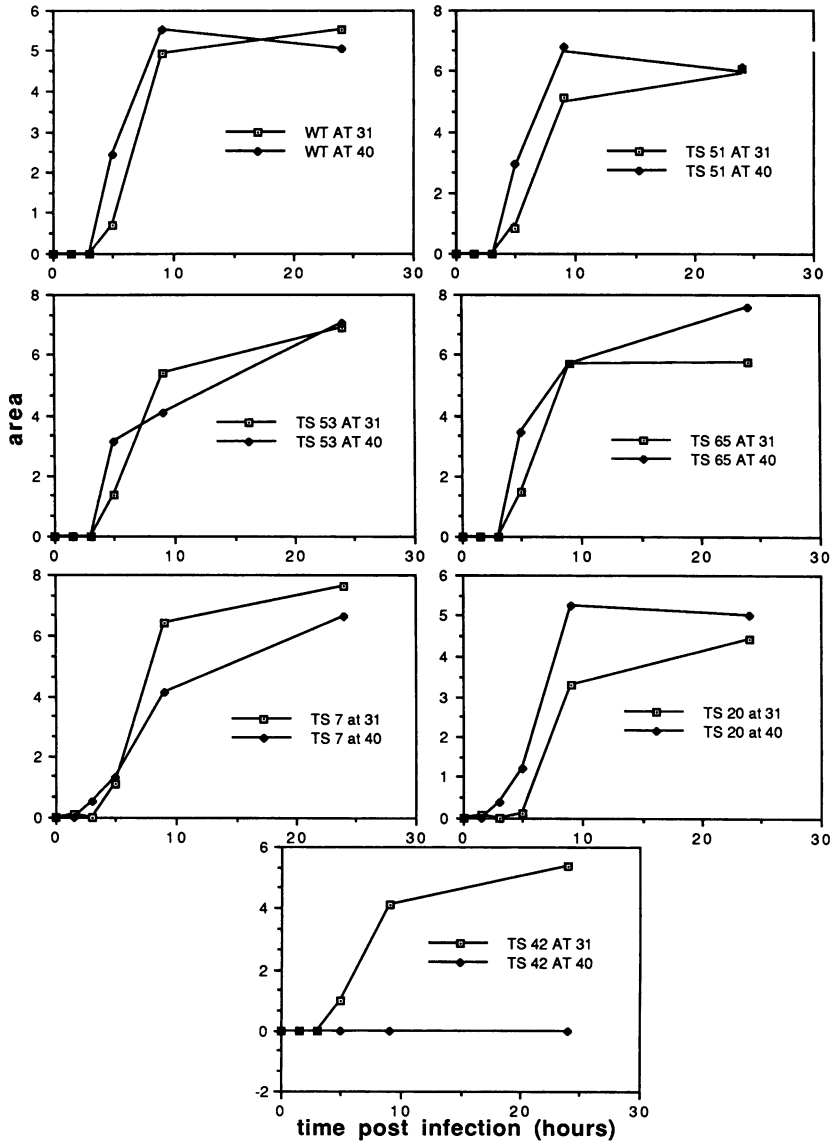
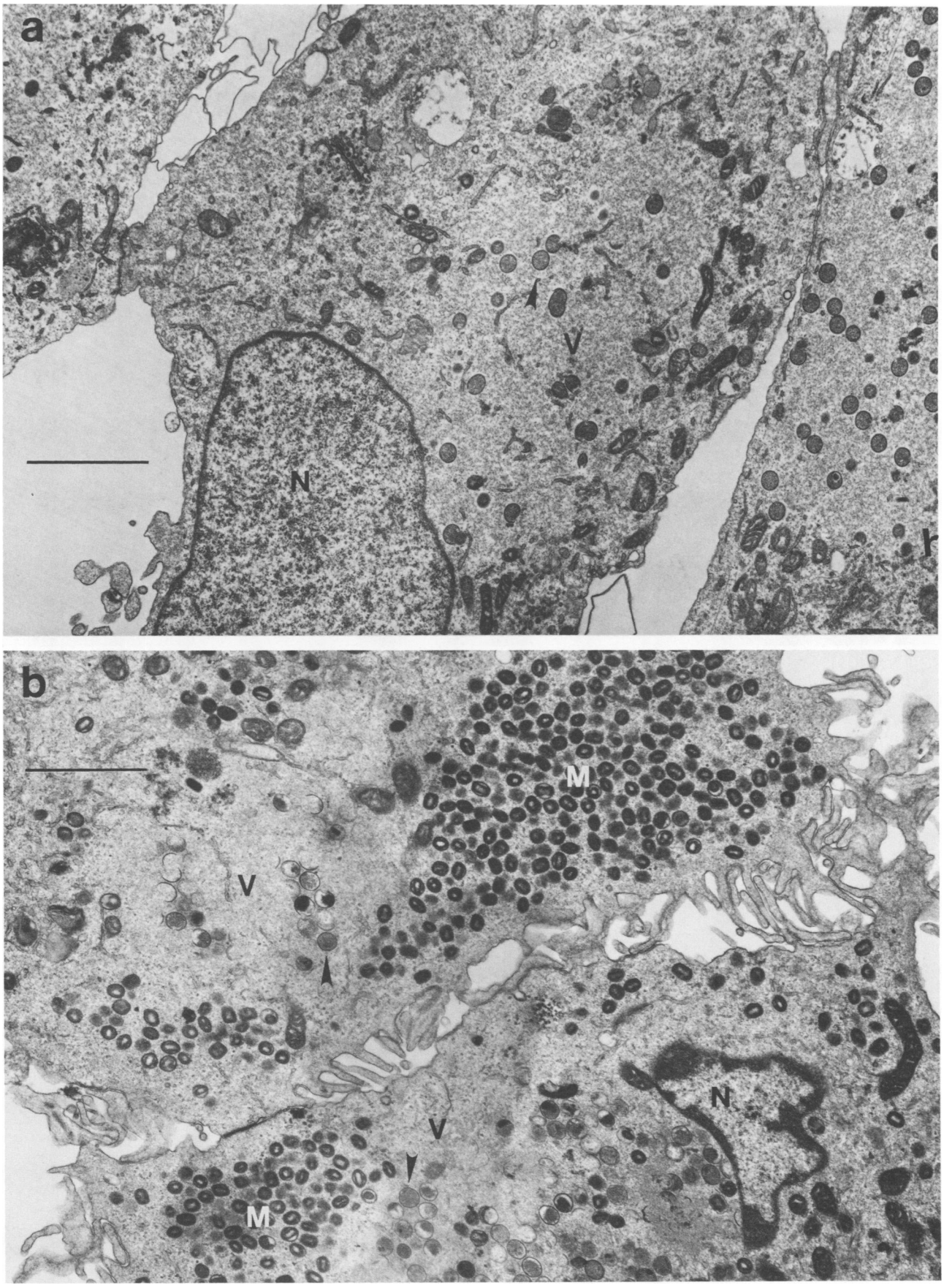


FIG. 5. Densitometric analysis of virus-specific DNA accumulation. The autoradiogram shown in Fig. 4 and another autoradiogram of the results of DNA accumulation in *ts*7- and *ts*20-infected cells (results not shown) at 31 or 40°C were scanned, using an LKB UltroScan XL laser densitometer. A graphical representation of the analysis is shown.

normal and virus particles in various stages of maturation. Areas of viroplasm at distinctly different stages of maturation can be seen. Some areas contains mostly immature particles, and mature particles are found in large clusters. In cells infected with wt virus for 24 h at 31°C (Fig. 6a), virus morphogenesis was less advanced than at 40°C. All cells exhibited areas of viroplasm containing immature particles. Clusters of mature particles could be found (results not shown) but they occurred less frequently at 31°C than at 40°C.

Electron microscopic images of infections with three RNA polymerase mutants, *ts*53, *ts*7, and *ts*51 are shown in Fig. 6c through h. In all cases (*ts*7 [Fig. 6c], *ts*53 [Fig. 6e], and *ts*51 [data not shown]), the appearance of mutant-infected cells incubated at 31°C was comparable with that of wt virus-infected cells at 31°C. Most importantly, every infected cell

FIG. 6. Electron microscopic analysis of wt- and mutant virus-infected cells. Cells were infected and processed for electron microscopy as described in Materials and Methods. (a) wt infection, 24 h postinfection, 31°C. Bar, 2 μm. (b) wt infection, 24 h postinfection, 40°C. Bar, 2 μm. (c) *ts*7 infection, 24 h postinfection, 31°C. Bar, 2 μm. (d) *ts*7 infection, 24 h postinfection, 40°C. The leftmost cell is mitotic. It has no nuclear membrane, and the chromosomes are condensed. Bar, 4 μm. (e) *ts*53 infection, 24 h postinfection, 31°C. Bar, 3 μm. (f) *ts*53 infection, 24 h postinfection, 40°C. Bar, 3 μm. (g) *ts*53 infection. Cells were incubated at 40°C for 24 h and then at 31°C for 24 h. Bar, 2 μm. (h) *ts*51 infection, 12 h postinfection, 40°C. Bar, 0.5 μm. V, Viroplasm; M, mature particles; N, nucleus; np, viral nucleoprotein. Arrowheads indicate immature virus particles.


a
N
V
b
M
V
V
N
M

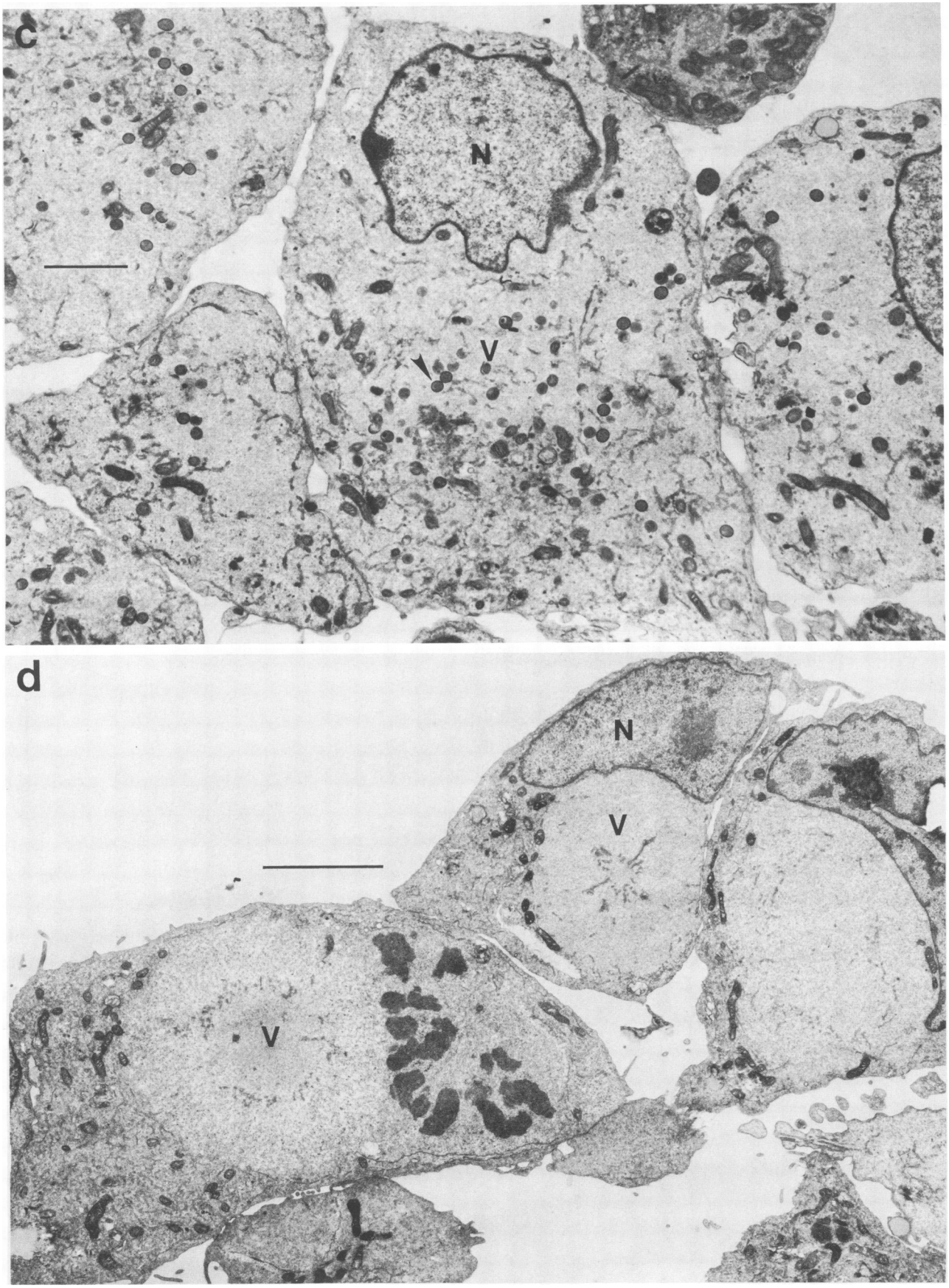


FIG. 6—*Continued.*

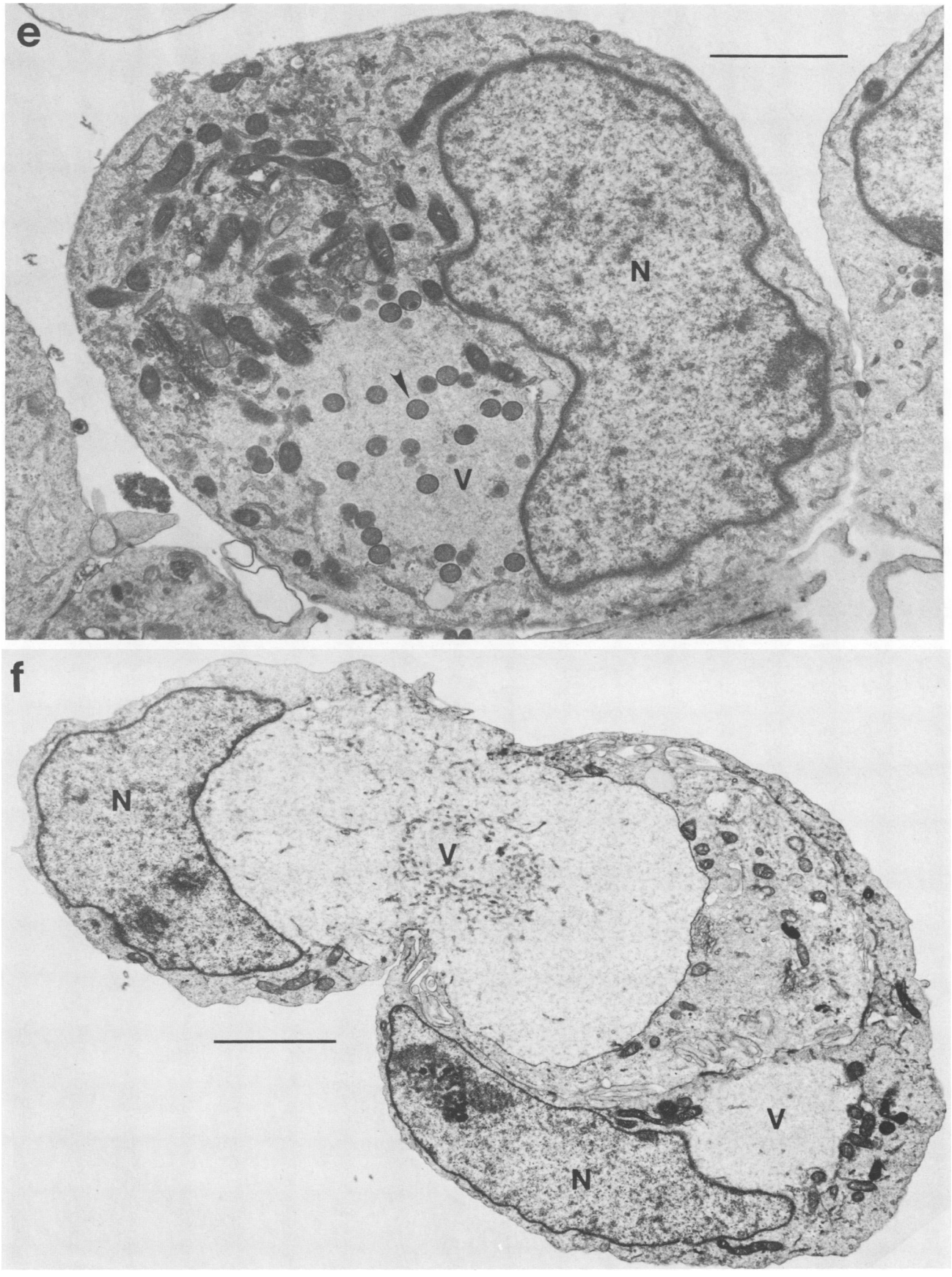


FIG. 6—*Continued.*

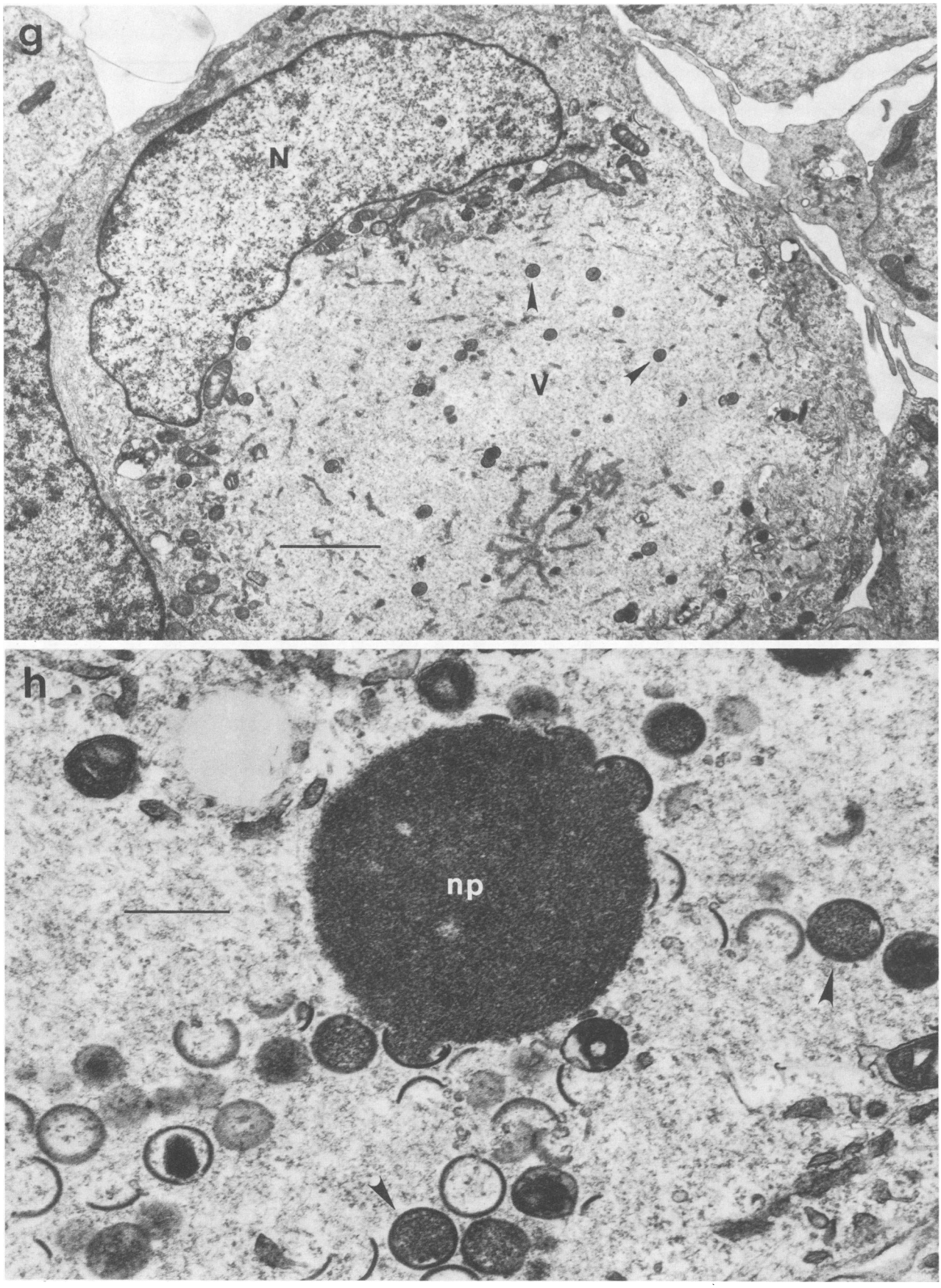


FIG. 6—*Continued.*

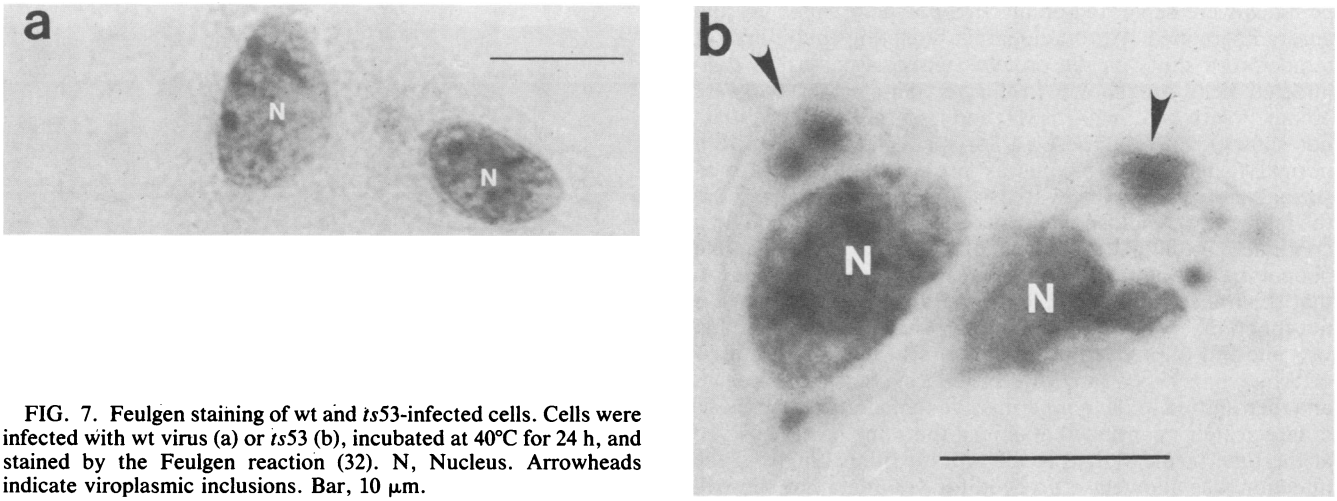


FIG. 7. Feulgen staining of wt and *ts*53-infected cells. Cells were infected with wt virus (a) or *ts*53 (b), incubated at 40°C for 24 h, and stained by the Feulgen reaction (32). N, Nucleus. Arrowheads indicate viroplasmic inclusions. Bar, 10 μm.

contained viroplasm with maturing virus particles, and clusters of mature particles could be found. When infected cells were incubated at 40°C, a strikingly different picture was obtained. In *ts*7-infected cells (Fig. 6d), virtually every cell contained at least one (sometimes more) inclusion of viroplasm (see below) which was fairly uniform in density, lighter in density than the surrounding cytoplasm, and devoid of either normal cellular organelles, viral membranes, or virus particles at any stage of maturation. These inclusions typically contained some membranes that did not have the appearance of viral membranes. No other evidence of viral morphogenesis could be found in these cells. At 40°C, *ts*53-infected cells were indistinguishable from *ts*7-infected cells (Fig. 6f). Cells infected with *ts*51 at 40°C (Fig. 6h) yielded electron microscopic images which were different than either wt-infected cells or cells infected with *ts*7 or *ts*53. In *ts*51-infected cells, viroplasmic inclusions were found which contained immature particles. In addition, these inclusions contained large, dense masses of viral nucleoprotein, often in close association with immature viral membranes. These structures were not observed in the corresponding wt infections. Few, if any, mature particles were observed in *ts*51-infected cells.

Two different experiments were done in order to prove that the inclusions observed in *ts*7- and *ts*53-infected cells at the nonpermissive temperature were viroplasm. First, *ts*53-infected cells which had been incubated at 40°C for 24 h were shifted down to 31°C for an additional 24 h, and sections from the cells were examined in the electron microscope (Fig. 6g). In these cells, we observed inclusions which were similar in appearance to the inclusions observed at 40°C, except that they now contained immature virus particles. We interpret this result to mean that the inclusions observed after an infection at the nonpermissive temperature are virus factories in which virions can develop when the temperature is lowered. In a second experiment, infected cells were fixed, stained by the Feulgen reaction (32), which is specific for DNA, and examined by light microscopy. Cells infected with either wt virus or *ts*53 at 31°C (results not shown) or with wt virus at 40°C (Fig. 7a) were similar in appearance. The only prominent stained structure in these cells was the nucleus. In contrast, cells infected with *ts*53 at 40°C exhibited large, stained cytoplasmic inclusions in addition to stained nuclei. We equate these cytoplasmic inclusions with the inclusions observed in the electron microscope. The fact that they stain by Feulgen reaction is evidence that they contain DNA. Apparently, any viroplasmic structures formed under conditions permissive for virus growth were too small to be resolved by this technique.

Effect of temperature shifts. We expected initially that mutants with *ts* lesions in a vaccinia virus RNA polymerase subunit would be defective in both early and late viral gene expression. Contrary to expectations, the data presented above show that five *ts* mutants which have lesions in two different RNA polymerase subunits were normal in early viral gene expression and only defective in late viral gene expression. These results suggest a model (see Fig. 10 and Discussion) in which two functionally distinct transcription complexes are used during the course of a vaccinia virus infection. Specifically, we propose that the virion-associated transcription complex is made up in part by an RNA polymerase assembled from subunits synthesized late during a viral infection and that this is the only RNA polymerase which can transcribe early viral genes. We further propose that a functionally distinct transcription complex, which we will call the cytoplasmic complex, is made up in part by a virus-encoded RNA polymerase assembled from subunits synthesized early during infection and that this cytoplasmic RNA polymerase transcribes only late viral genes. Finally, we propose that the *ts* mutants are defective in the assembly of a functional transcription complex at the nonpermissive temperature but that once assembled at a permissive temperature, a transcription complex containing an RNA polymerase with a mutant subunit is not thermolabile. Thus, when a cell is infected with an RNA polymerase mutant, early viral transcription proceeds from a virion-associated transcription complex which was assembled during growth of the virus at the permissive temperature, and late transcription is defective because the cytoplasmic transcription complex cannot be assembled at the nonpermissive temperature. One prediction of this model is that if a mutant cytoplasmic transcription complex is allowed to assemble at the permissive temperature, it should continue to function when shifted to a nonpermissive temperature. To test this prediction, we measured the effect of temperature shifts on late viral protein synthesis in each of the mutants.

Cells were infected at the permissive temperature and at various times postinfection were moved to the nonpermissive temperature. At various times after the shifts, the cells were pulse-labeled with [^{35}S]methionine, the labeled poly-

peptides were separated on an SDS-polyacrylamide gel and autoradiographed. Autoradiograms depicting the effects of temperature shifts on the protein synthesis pattern in *ts*53-infected cells are shown in Fig. 8. Similar results were obtained with *ts*7-, *ts*20-, *ts*51-, and *ts*65-infected cells (data not shown). *ts*53 synthesized early and late proteins in a normal fashion at 31°C but as previously observed was strongly defective in the synthesis of late proteins at 40°C. If the infection was allowed to proceed for 4 h at 31°C and then continued at 40°C, the virus exhibited a defective late phenotype (Fig. 8a). We interpret this observation to mean that the transcription complex that transcribes late genes is not functional at 4 h after infection. When the *ts*53 infection was allowed to proceed at the permissive temperature for 6 or 8 h, a time when late viral protein synthesis has begun, and then shifted to the nonpermissive temperature, synthesis of late proteins continued at almost the same level observed at the time of the shift (Fig. 8b and c). Interestingly, if the infection was allowed to proceed for 4, 6 (data not shown), or 8 h (Fig. 9) at 40°C, when no late viral protein synthesis was observed, and then continued at the permissive temperature, late viral protein synthesis resumed after a lag of 1 to 2 h.

Our interpretation of the temperature shift up experiment is predicated on the assumption that the labeling of virus proteins reflects active transcription, rather than translation of stable late messages. To test this assumption, we studied the effect of temperature shift up on late viral protein synthesis in the presence of ActD. The experiment was done in parallel with the temperature shift experiment done in the absence of ActD in *ts*53-infected cells (Fig. 8). ActD was added to *ts*53-infected cells at 4, 6, and 8 h postinfection at 31°C, and the dishes were then moved to 40°C. At regular intervals (Fig. 8), the infected cells were pulse-labeled with [^{35}S]methionine. The labeled polypeptides were separated on an SDS-polyacrylamide gel and visualized by autoradiography. The data showed that in the presence of ActD, late viral protein synthesis in *ts*53-infected cells diminished rapidly, indicating that late viral messages were unstable in *ts*53-infected cells. A similar result was obtained for late wt viral mRNAs. The half-lives of wt late viral mRNAs at 40°C were determined to be between 45 min and 1 h (data not shown), which is in agreement with previous observations (30, 37).

On the basis of these data, we conclude that the continued late protein synthesis that we see in *ts*53-infected cells at 2 and 4 h after shifting the temperature up late during infection represents transcription by a complex which was assembled at the permissive temperature and retains function at the nonpermissive temperature. These results support our proposition that the molecular defect in these mutants lies in their inability to assemble at the nonpermissive temperature a functional cytoplasmic transcription complex that transcribes late viral genes.

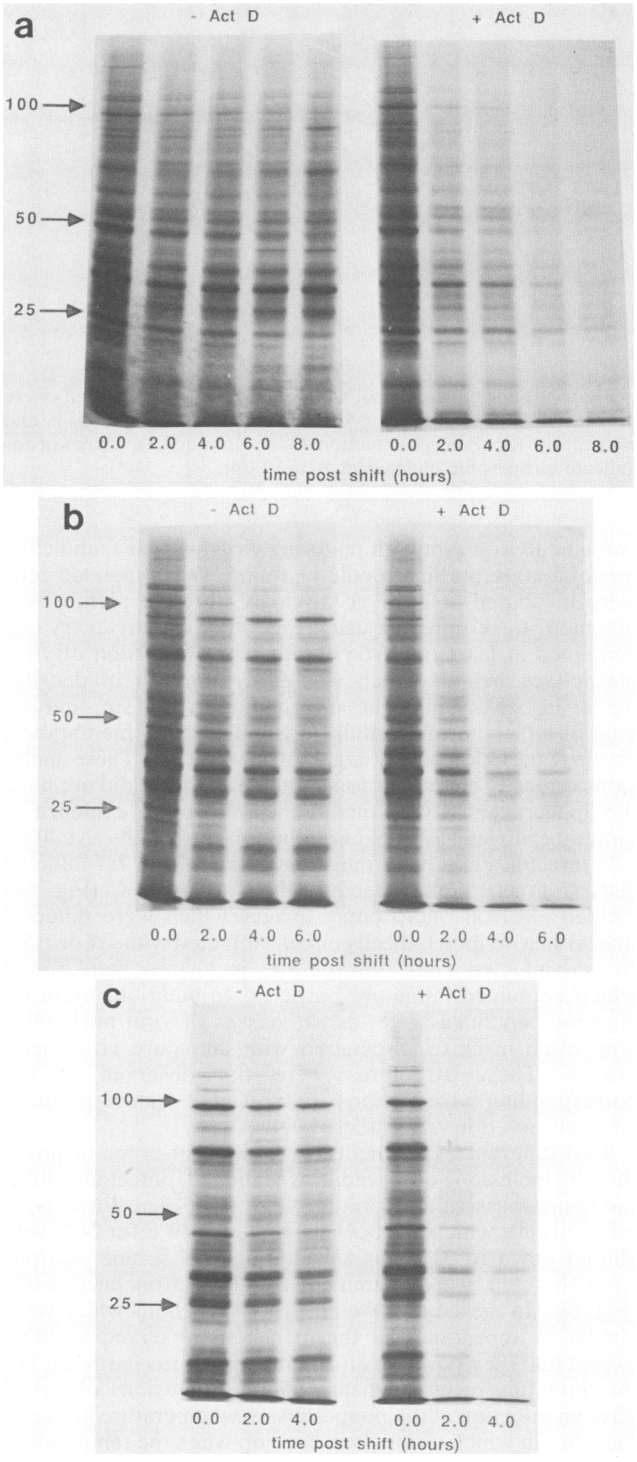


FIG. 8. Effect of a temperature shift up on viral protein synthesis, with and without ActD in *ts*53-infected cells. BSC40 cells were infected with *ts*53 at 31°C. At 4 (a), 6 (b), and 8 (c) h postinfection, the infected cells were shifted to 40°C in the absence (− Act D) or the presence (+ Act D) of ActD. At various times after the shift, indicated at the bottom of each autoradiogram, the infected cells were pulse-labeled with [^{35}S]methionine. The labeled polypeptides were separated on a 10% SDS-polyacrylamide gel and autoradiographed. The autoradiograms are shown. Approximate molecular masses (in kilodaltons) are indicated to the left.

DISCUSSION

We initially expected that mutants with *ts* lesions in vaccinia virus RNA polymerase subunits would be defective in both early and late viral gene expression and therefore be defective in viral DNA replication and virion morphogenesis as well. However, our data show that five mutants with *ts* lesions in either the 22- or 147-kDa subunit of the viral RNA polymerase were normal in host shutoff, initiation of early viral gene expression, and viral DNA synthesis. The mutants were defective in the shutoff of early viral gene expression,

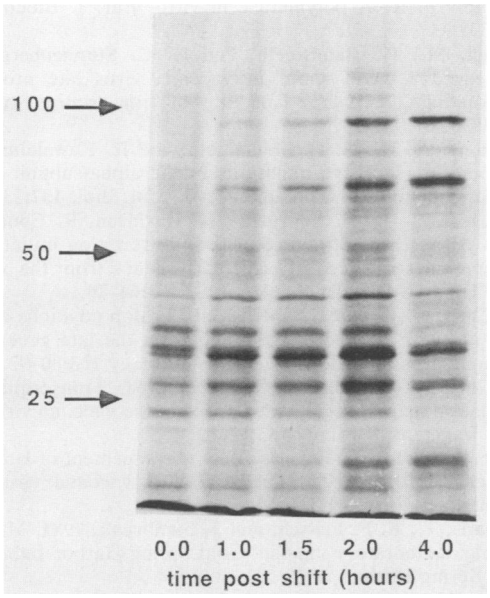


FIG. 9. Effect of a temperature shift down on viral protein synthesis in *ts*53-infected cells. BSC40 cells were infected at 40°C with *ts*53. At 8 h postinfection, the infected cells were shifted to 31°C. At various times after the shift, indicated at the bottom of each autoradiogram, the infected cells were pulse-labeled with [^{35}S]methionine. The labeled polypeptides were separated on a 10% SDS-polyacrylamide gel and autoradiographed. The autoradiograms are shown. Approximate molecular masses (in kilodaltons) are indicated to the left.

initiation of late viral gene expression, and virion morphogenesis.

The data presented here, in conjunction with in vitro transcription studies (see Introduction) and previous characterization of vaccinia virus RNA polymerase mutants (14), support a model for control of vaccinia virus gene expression (Fig. 10) in which two functionally distinct transcription complexes are active during a vaccinia virus infection. One complex transcribes only early viral genes, and another transcribes only late viral genes. The phenotypic characterization of RNA polymerase mutants reported here shows that the mutant subunits selectively affected the late transcription complex, even though in a wt infection, the gene products were found in both virion- and cell-derived RNA polymerase.

Our interpretation of the phenotype of the RNA polymerase mutants is predicated on the understanding that *ts* mutations generally fall into two classes, mutants which are thermolabile (*tl*) and mutants which show temperature-sensitive synthesis (*tss*) (34, 46). *tl* mutants encode gene products which, once formed correctly at the permissive temperature, lose function if raised to the nonpermissive temperature. *tss* mutants are temperature sensitive in the folding or association of mutant protein subunits during synthesis. If the gene product from a *tss* mutant is allowed to form at a permissive temperature, it does not lose function when raised to the nonpermissive temperature. Our results show that early viral transcription is normal during infection with vaccinia virus RNA polymerase *ts* mutants and that late viral transcription continues after a temperature shift up late during a mutant infection. This eliminates the possibility that these are *tl* mutants and therefore strongly suggests that the mutants are *tss* mutants, that is, that they are defective in the

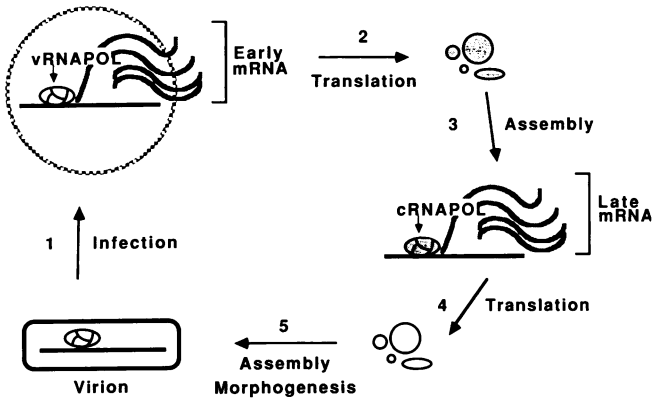


FIG. 10. Model for the cycle of RNA polymerase synthesis and function during vaccinia virus infection. vRNAPOL, virion RNA polymerase; cRNAPOL, cytoplasmic RNA polymerase.

assembly of a functional RNA polymerase at the nonpermissive temperature. Since the mutants are *tss* mutants and since they specifically affect late transcription, we conclude that early viral transcription is directed entirely by the virion transcription complex, and that formation of a functional late viral transcription complex requires de novo synthesis of viral RNA polymerase during infection. We are testing this further using antibodies directed against the RNA polymerase subunits. Although *tl* mutants of *Saccharomyces cerevisiae* RNA polymerase have been characterized (29), most RNA polymerase mutants are *tss* mutants (16, 18).

The morphogenetic defect for all the strongly defective late mutants is characterized by the presence of large DNA-containing viroplasmic inclusions devoid of viral membranes or virion precursor particles. This phenotype follows logically from the absence of late viral protein synthesis. It is interesting to note that the viral DNA which accumulates in cells infected with these and other defective late mutants contains an abnormally high fraction of concatemeric molecules (Mike Merchlinsky and Bernard Moss, submitted for publication; L. DeLange, manuscript in preparation).

The RNA polymerase mutants studied here do not all have identical phenotypes. *ts*53 and *ts*51 provide an extreme comparison. Both are tight *ts* mutants in the 147-kDa subunit of the viral RNA polymerase, yet while *ts*53 exhibits almost no late viral protein synthesis, late gene expression in a *ts*51 infection occurs at almost normal levels and is only slightly delayed relative to a wt infection. Since shutoff of early viral protein synthesis seems to require late viral protein synthesis (7, 8), it seems likely that the apparently normal early shutoff observed during a *ts*51 infection results from the almost normal synthesis of late viral proteins. Whereas virion morphogenesis is absent in a *ts*53 infection at the nonpermissive temperature, morphogenesis in a *ts*51 infection proceeds to an intermediate and possibly abnormal stage. It is obvious that the transcriptional defect in *ts*53 can account for temperature sensitivity of virus growth. In contrast, while the primary defect in *ts*51 is undoubtedly transcriptional, the phenotype is much more subtle and is manifested primarily as a defect in virion morphogenesis. It seems likely that the transcriptional defect results in reduced synthesis of component(s) essential for normal morphogenesis. Ensinger has observed a similar heterogeneity of phenotype in vaccinia virus RNA polymerase mutants (14).

The phenotype of vaccinia virus RNA polymerase mutants provides us with initial evidence that late genes are

transcribed by a newly synthesized RNA polymerase. This process represents one of the mechanisms controlling the switch from early to late vaccinia virus gene expression. The mutants will therefore prove useful in understanding interactions of the core RNA polymerase with other transcription complex factors that attribute specificity to the core polymerase in recognizing early and late viral genes.

ACKNOWLEDGMENTS

We thank Ted Szczesny, Bob Summers, and Barry Eckert for instruction and continuing advice in electron microscopy. We thank Ed Niles and Zahra Fathi for helpful discussions while this work was in progress.

This work was supported by Public Health Service grant AI18094 from the National Institutes of Health.

LITERATURE CITED

1. **Baroudy, B. M., and B. Moss.** 1980. Purification and characterization of a DNA-dependent RNA polymerase from vaccinia virus. J. Biol. Chem. **255**:4372–4380.
2. **Bertholet, C., P. Stocco, E. Van Meir, and R. Wittek.** 1986. Functional analysis of the 5′ flanking sequence of a vaccinia virus late gene. EMBO J. **5**:1951–1957.
3. **Bertholet, C., E. Van Meir, B. ten Heggeler-Bordier, and R. Wittek.** 1987. Vaccinia virus produces late mRNAs by discontinuous synthesis. Cell **50**:153–162.
4. **Broyles, S. S., and B. Moss.** 1986. Homology between RNA polymerases of poxviruses, prokaryotes, and eukaryotes: nucleotide sequence and transcriptional analysis of vaccinia virus genes encoding 147-kDa and 22-kDa subunits. Proc. Natl. Acad. Sci. USA **83**:3141–3145.
5. **Broyles, S. S., and B. Moss.** 1987. Sedimentation of an RNA polymerase complex from vaccinia virus that specifically initiates and terminates transcription. Mol. Cell. Biol. **7**:7–14.
6. **Cochran, M. A., C. Puckett, and B. Moss.** 1985. In vitro mutagenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signal. J. Virol. **54**:30–37.
7. **Condit, R. C., and A. Motyczka.** 1981. Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. Virology **113**:224–241.
8. **Condit, R. C., A. Motyczka, and G. Spizz.** 1983. Isolation, characterization, and physical mapping of temperature-sensitive mutants of vaccinia virus. Virology **128**:429–443.
9. **Coupar, B. E. H., D. B. Boyle, and G. W. Both.** 1987. Effect of in vitro mutations in a vaccinia virus early promoter region monitored by herpes simplex virus thymidine kinase expression in recombinant vaccinia virus. J. Gen. Virol. **68**:2299–2309.
10. **Dales, S.** 1963. The uptake and development of vaccinia virus in strain L cells followed with labeled viral deoxyribonucleic acid. J. Cell Biol. **18**:51–72.
11. **Dales, S., V. Milovanivitch, B. Pogo, S. Weintraub, T. Huima, S. Wilton, and G. McFadden.** 1978. Biogenesis of vaccinia: isolation of conditional lethal mutants and electron microscopic characterization of their phenotypically expressed defects. Virology **84**:403–428.
12. **Dales, S., and E. Mosbach.** 1968. Vaccinia as a model for membrane biogenesis. Virology **35**:546–583.
13. **Drillien, R., F. Tripier, F. Koehren, and A. Kirn.** 1977. A temperature-sensitive mutant of vaccinia virus defective in an early stage of morphogenesis. Virology **79**:369–380.
14. **Ensinger, M. J.** 1987. Phenotypic characterization of temperature-sensitive mutants of vaccinia virus with mutations in a 135,000-M_r subunit of the virion-associated DNA-dependent RNA polymerase. J. Virol. **61**:1842–1850.
15. **Essani, K., R. Dugre, and S. Dales.** 1982. Biogenesis of vaccinia: involvement of spicules of the envelope during virion assembly examined by means of conditional lethal mutants and serology. Virology **118**:279–282.
16. **Gross, G. C., D. A. Fields, and E. K. F. Bautz.** 1977. Temperature-sensitive mutants of Escherichia coli with defects in the assembly of RNA polymerase in vitro. Eur. J. Biochem. **81**: 333–338.
17. **Hanggi, M., W. Bannwarth, and H. G. Stunnenberg.** 1986. Conserved TAAAT motif in vaccinia virus late promoters: overlapping TATA box and site of transcription initiation. EMBO J. **5**:1071–1076.
18. **Ishihama, A. M., A. H. Shimamoto, and K. Kawakami.** 1980. Temperature-sensitive mutations in the alpha-subunit gene of Escherichia coli RNA polymerase. J. Mol. Biol. **137**:137–150.
19. **Lee-Chen, G. J., N. Bourgeois, K. Davidson, R. Condit, and E. G. Niles.** 1988. Structure of the transcription initiation and termination sequences of eight early genes from the vaccinia virus Hind III D fragment. Virology **163**:64–79.
20. **Lee-Chen, G. J., and E. G. Niles.** 1988. Map positions of the 5′ ends of eight mRNAs synthesized from the late genes in the vaccinia virus HindIII D fragment. Virology **163**:80–92.
21. **Lee-Chen, G. J., and E. G. Niles.** 1988. Transcription and translation map of the thirteen genes in the vaccinia virus Hind III D fragment. Virology **163**:52–63.
22. **Mahr, A., and B. E. Roberts.** 1984. Arrangement of late RNAs transcribed from a 7.1-kilobase *Eco*RI vaccinia virus DNA fragment. J. Virol. **49**:510–520.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1983. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **McDonald, K.** 1984. Osmium ferricyanide fixation improves microfilament preservation and membrane visualization in a variety of animal cell types. J. Ultrastruct. Res. **86**:107–118.
25. **Miner, J. N., S. L. Weinrich, and D. E. Hruby.** 1988. Molecular dissection of *cis*-acting regulatory elements from 5′-proximal regions of a vaccinia virus late gene cluster. J. Virol. **62**: 297–304.
26. **Morrison, D. K., and R. W. Moyer.** 1986. Detection of a subunit of cellular Pol II within highly purified preparations of RNA polymerase isolated from rabbit poxvirus virions. Cell **44**: 587–596.
27. **Moss, B.** 1985. Replication of poxviruses, p. 685–703. *In* B. N. Fields (ed.), Virology. Raven Press, New York.
28. **Niles, E. G., R. C. Condit, P. Caro, K. Davidson, L. Matusick, and J. Seto.** 1986. Nucleotide sequence and genetic map of the 16-kb vaccinia virus HindIII D fragment. Virology **153**:96–112.
29. **Nonet, M., C. Scafe, J. Sexton, and R. Young.** 1987. Eucaryotic RNA polymerase conditional mutant that rapidly ceases mRNA synthesis. Mol. Cell. Biol. **7**:1602–1611.
30. **Oda, K., and W. K. Joklik.** 1967. Hybridization and sedimentation studies on "early" and "late" vaccinia messenger RNA. J. Mol. Biol. **27**:395–419.
31. **Pacha, R. F., and R. C. Condit.** 1985. Characterization of a temperature-sensitive mutant of vaccinia virus reveals a novel function that prevents virus-induced breakdown of RNA. J. Virol. **56**:395–403.
32. **Putt, F. A.** 1972. The Feulgen reaction to demonstrate thymonucleic acid (DNA), p. 96–97. *In* The manual of histopathological staining methods. John Wiley & Sons, Inc., New York.
33. **Rohrmann, G., L. Yuen, and B. Moss.** 1986. Transcription of vaccinia virus early genes by enzymes isolated from vaccinia virions terminates downstream of a regulatory sequence. Cell **46**:1029–1035.
34. **Sadler, J. R., and A. Novick.** 1965. The properties of repressors and the kinetics of its action. J. Mol. Biol. **12**:305–327.
35. **Schwer, B., and H. G. Stunnenberg.** 1988. Vaccinia virus late transcripts generated in vitro have a poly(A) head. EMBO J. **7**:1183–1190.
36. **Schwer, B., P. Visca, J. C. Vos, and H. G. Stunnenberg.** 1987. Discontinuous transcription or RNA processing of vaccinia virus late messengers results in a 5′ poly(A) leader. Cell **50**:163–169.
37. **Sedbring, E. D., and N. P. Salzman.** 1967. Metabolic properties of early and late vaccinia messenger RNA. J. Virol. **1**:550–558.
38. **Selden, R. F.** 1987. Analysis of RNA by Northern hybridization, 4.9.1-4.9.7. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Greene Publishing Associates

and John Wiley & Sons, Inc.
39. **Shuman, S., S. S. Broyles, and B. Moss.** 1987. Purification and characterization of a transcription termination factor from vaccinia virions. J. Biol. Chem. **262**:12372–12380.
40. **Silver, M., and S. Dales.** 1982. Biogenesis of vaccinia: interrelationship between post-translational cleavage, virus assembly, and maturation. Virology **117**:341–356.
41. **Spencer, E., S. Shuman, and J. Hurwitz.** 1980. Purification and properties of the vaccinia DNA-dependent RNA polymerase. J. Biol. Chem. **255**:5388–5395.
42. **Spurr, A. R.** 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**:31–43.
43. **Thompson, C. L., U. Hooda-Dhingra, and R. C. Condit.** 1989. Fine structure mapping of five temperature-sensitive mutants in the 22- and 147-kilodalton subunits of vaccinia virus DNA-dependent RNA polymerase. J. Virol. **63**:705–713.
44. **Weir, J. P., and B. Moss.** 1987. Determination of the promoter region of an early vaccinia virus gene encoding thymidine kinase. Virology **158**:206–210.
45. **Weir, J., and B. Moss.** 1987. Determination of the transcriptional regulatory region of a vaccinia virus late gene. J. Virol. **61**:75–80.
46. **Yu, M., and J. King.** 1988. Surface amino acids as sites of temperature-sensitive folding mutations in the P22 tail spike protein. J. Biol. Chem. **263**:1424–1431.
47. **Yuen, L., and B. Moss.** 1987. Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. Proc. Natl. Acad. Sci. USA **84**:6417–6421.