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Temperature-Sensitive Mutations in the Vaccinia Virus H4 Gene Encoding a Component of the Virion RNA Polymerase

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Four previously isolated temperature-sensitive (*ts*) mutants of vaccinia virus WR (*ts*1, *ts*31, *ts*55, and *ts*58) comprising a single complementation group (R. C. Condit, A. Motyczka, and G. Spizz, Virology 128:429–443, 1983) have been mapped by marker rescue to the H4L open reading frame located within the genomic *Hin*dIII-H DNA fragment. The H4 gene is predicted to encode a 93.6-kDa polypeptide expressed at late times during infection. Nucleotide sequence alterations responsible for thermolabile growth lead to amino acid substitutions in the H4 gene product. All four *ts* alleles display "normal" patterns of early and late viral protein synthesis at the nonpermissive temperature (40°C). Mature virion particles, microscopically indistinguishable from wild-type virions, are produced in the cytoplasm of cells infected with *ts*1 at 40°C. Western immunoblot analysis localizes the H4 protein to the virion core. After solubilization from cores, the H4 protein is associated during purification with transcriptionally active vaccinia virus DNA-dependent RNA polymerase.

Vaccinia virus, a member of the poxvirus family, replicates in the cytoplasm of mammalian cells. Nuclear independence is reflected in the dedication of a considerable portion of the large viral DNA genome to the encoding of enzymes involved in viral mRNA synthesis and viral DNA replication (26). The mRNA synthesis machinery is encapsidated within the infectious virus particle, where it is poised to specifically transcribe the early class of viral genes (27). The enzymatic content of the virion, its large size, and its unique morphology (8, 26) are features that contribute to a challenging research model for virus development. Although many virion components have been characterized, the molecular details of virus structure remain obscure.

The nucleotide sequence of the 192,000-bp vaccinia virus genome predicts that the virus encodes 198 polypeptides (18). At least 55 genes at the left and right ends of the genome are dispensable for virus growth in cell culture (30). Genes essential for virus growth have been defined through classical genetic approaches, i.e., via the study of conditionally lethal (temperature-sensitive [*ts*]) mutations (9–11, 13, 15, 41). The mutant collection of R. Condit and colleagues, consisting of 65 *ts* mutants grouped into 31 complementation groups, has been classified with respect to the patterns of macromolecular synthesis under nonpermissive growth conditions (9, 10, 41). Four phenotypes have been described, including (i) DNA replication negative, (ii) abortive late protein synthesis, (iii) defective late protein synthesis, and (iv) "normal." Normal mutants exhibit a wild-type (WT) pattern of DNA replication and protein synthesis at the nonpermissive temperature.

The normal phenotype is suggestive of mutation of a viral gene whose protein product might either participate in virion morphogenesis or be essential for establishment of the next round of infection. Several of the normal mutants have been mapped by marker rescue to individual viral genes. These include (i) the D2L and D3R genes, both expressed late in infection, which respectively encode 17-kDa and 28-kDa virion structural proteins that colocalize to the virus core (14, 29, 36); (ii) gene D13L, encoding a 62-kDa late protein implicated in virus morphogenesis (3, 23, 36, 40); (iii) gene D6R, encoding the 73-kDa subunit of VETF, an early transcription factor which is synthesized late in infection and packaged into the virus core for the synthesis of early mRNAs in the next round of infection (5, 36); and (iv) gene I8R, expressed at both early and late times, which encodes a 78-kDa virion component (16). Thermolabile mutations in the I8 gene result in the production of progeny virions that are morphologically normal but noninfectious (17).

In this article, we present a molecular analysis of four mutant viruses (*ts*1, *ts*31, *ts*55, and *ts*58) shown by Condit and Motyczka (9) to constitute a single complementation group (group 28). The genetic lesions conferring the growth defect have been mapped by marker rescue to the vaccinia virus H4 gene and shown to be point mutations resulting in amino acid substitutions at the protein level. All four *ts* mutants display a normal phenotype for macromolecular synthesis at the nonpermissive temperature. Electron microscopic analysis of the *ts*1 mutant suggests a role for the H4 gene product in infectivity rather than morphogenesis. The H4 gene product is shown to be a component of the virion core that is associated during purification with the viral DNA-dependent RNA polymerase. Identification of the H4 protein as a polypeptide associated with vaccinia virus RNA polymerase has been reported by Ahn and Moss (1).

MATERIALS AND METHODS

Cells and viruses. BSC40 cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal calf serum (FCS). WT vaccinia virus WR, obtained from B. Moss, National Institutes of Health, was propagated in BSC40 cells grown at 37°C. Thermosensitive mutants (*ts*1, *ts*31, *ts*55, and *ts*58) were kindly provided to us by R. Condit, University of Florida. Mutant viruses were propagated in BSC40 cells at 31°C (permissive temperature). The mutant viruses were amplified twice in the course of these studies. The thermolability of mutant virus stocks was verified at each passage by comparative titration on BSC40 cells at 31 and 40°C (nonpermissive temperature).

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One-step growth. Confluent monolayers of BSC40 cells (35-mm dishes) maintained at either 31 or 40°C were infected with either WT or mutant virus at a multiplicity of infection (MOI) of 5. The inoculum was removed after 30 min, and the cells were washed once with medium and then overlaid with fresh DME–5% FCS. Infected cells were harvested at various times postinfection by scraping the monolayer with a Teflon policeman. Cells were pelleted in a clinical centrifuge and then resuspended in 1 ml of DME. The suspension was subjected to three cycles of freezing and thawing followed by three 30-s bursts of sonication. Titers were determined by serial 10-fold dilution of virus onto BSC40 cells grown at 31°C.

Metabolic labeling. Confluent BSC40 monolayers were infected with virus at an MOI of 10 at 31 and 40°C. At various times postinfection, the medium was removed, and the cells were washed with methionine-free DME and then overlaid with methionine-free DME containing 30 μCi of [^{35}S]methionine (>800 Ci/mmol) per ml for 30 min. This medium was removed, and the cells were lysed in situ by the addition of 0.15 ml of a solution containing 0.065 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 10% glycerol. Lysates were stored at −20°C. Samples (25-μl aliquots) were heated at 100°C for 5 min and then electrophoresed through a 12% polyacrylamide gel containing 0.1% SDS. Radiolabeled polypeptides were visualized by autoradiographic exposure of the dried gel.

Plasmids and molecular cloning. Plasmid pHH, containing the 8.6-kb *Hin*dIII-H restriction fragment of vaccinia virus WR cloned into pUC13, was kindly supplied by M. Merchlinsky, National Institutes of Health. Subclones of the H fragment deleted unidirectionally from the rightward end were generated by cleaving pHH with endonuclease *Nsi*I or *Sst*I, which cut at sites in the pUC polylinker and in the viral DNA insert. Digestion products were resolved by agarose gel electrophoresis. The fragment containing both vector and viral DNA was recovered from an excised gel slice by electroelution. This fragment was circularized by incubation with T4 DNA ligase, and the reaction products were transformed into *Escherichia coli* JM109. Plasmids pHN and pHS (named according to the restriction sites at the borders of the viral insert; see Fig. 2) were prepared by alkaline lysis and CsCl equilibrium centrifugation.

A plasmid containing only the H4 open reading frame (ORF) (18) was generated as follows. Oligonucleotide primers complementary to the 5′ and 3′ ends of the H4 ORF and containing restriction sites for *Nde*I and *Bam*HI, respectively, were used to amplify the H4 gene from the pHH plasmid. Polymerase chain reaction (PCR) was carried out with a GeneAmp reagent kit (Perkin Elmer Cetus). PCR products were gel purified and cleaved with endonucleases *Nde*I and *Bam*HI. This H4-containing DNA was then inserted into plasmid pET3c (35) which had been digested with *Nde*I and *Bam*HI to generate plasmid petH4.

The genomic *Hin*dIII fragments of WT and mutant viruses were cloned as follows. Virus was grown in 162-cm^2 flasks at 31°C. Viral DNA was prepared as described before (21). The *Hin*dIII digestion products of this DNA were resolved by preparative electrophoresis in a 0.6% agarose gel containing 0.5 μg of ethidium bromide per ml in TBE (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA). The *Hin*dIII-H fragment was visualized by UV transillumination, excised from the gel, and then recovered by electroelution. Each viral *Hin*dIII-H fragment was inserted into pUC19 that had been cleaved with *Hin*dIII. Plasmid DNA was isolated as described above.

Marker rescue. Confluent BSC40 cell monolayers (35-mm dishes) were infected with virus at an MOI of 5 at 31°C. The inoculum was removed after 30 min, and the cells were washed once with medium and overlaid with DME–5% FCS. After 30 min, cells were dislodged from the monolayer by treatment with 1 ml of 0.05% trypsin–0.53 mM EDTA. Suspended cells were mixed with 4 ml of DME and recovered by centrifugation for 5 min at 4°C in a clinical centrifuge. The pellet was washed with 0.5 ml of HBS (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.0], 150 mM NaCl, 0.7 mM Na_2HPO_4, 5 mM KCl, 6 mM dextrose) and centrifuged as before. The pellet was resuspended in 1 ml of cold HBS by pipetting and kept on ice. An aliquot (0.8 ml) of the suspension was transferred to a chilled Bio-Rad 0.4-cm electrode gap cuvette. Plasmid DNA (10 μg) was added to the cuvette (with mixing by pipetting), and the cuvette was chilled on ice for 10 min. The cuvette was pulsed at 200 V (capacitance, 960 μF) in a Bio-Rad Gene Pulser equipped with a Bio-Rad Capacitance Extender and then kept on ice for 10 min. The suspension was diluted into 8 ml of medium at room temperature. An aliquot (3 ml) was then applied to a confluent cell monolayer of BSC40 cells (35-mm well) maintained at 40°C. After 2 days of incubation at 40°C, the cells were stained with 0.1% crystal violet in order to visualize plaques formed from infectious centers.

DNA sequence analysis. Sequencing of plasmid DNA was performed by the dideoxy nucleotide chain termination method (20). A T7 DNA polymerase-based sequencing kit (Sequenase, Version 2.0) was used according to protocols supplied by the manufacturer (United States Biochemical).

Electron microscopy. Confluent BSC40 cell monolayers (35-mm-diameter dishes) maintained at either 31 or 40°C were infected with virus at an MOI of 5. The inoculum was removed after 1 h, and the cells were washed twice with medium and then overlaid with fresh DME–5% FCS. At 24 h postinfection, the medium was removed and replaced with 1 ml of 0.05% trypsin–0.53 mM EDTA. The detached cells were added to 4 ml of DME and then spun in a clinical centrifuge at 4°C. The supernatant was removed, and the cell pellet was placed immediately on ice. Cells were fixed initially in 2.5% glutaraldehyde and then in 2% osmium tetroxide. Specimens were dehydrated and then embedded in epoxy resin (Polybed 812). Thin sections were stained with uranyl acetate and lead citrate for visualization in a JEOL 1200 CX transmission electron microscope.

Anti-H4 serum. A synthetic peptide, KIYKNSFSEDHNN SLSD (corresponding to the predicted H4 coding sequence from amino acid positions 242 to 258), was coupled to ovalbumin and used to prepare rabbit antipeptide sera. Immunization was performed at Pocono Hill Rabbit Farm, Canadensis, Pa. The specificity of preimmune and immune sera was determined by Western immunoblotting against appropriate test antigens. Protein samples were electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. Polypeptides were transferred electrophoretically to a nitrocellulose membrane that was then blocked in TBST buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl, 0.05% Tween-20) containing 1% bovine serum albumin. Membranes were incubated for 30 min at room temperature with rabbit serum diluted in TBST. After removal of serum and washing with TBST, bound antibodies were localized by incubation with immunoglobulin conjugated with alkaline phosphatase by using a ProtoBlot AP system (Promega) according to the instructions of the manufacturer.

Virion extracts and enzyme purification. Vaccinia virions,

grown in HeLa cell suspension cultures and purified by sucrose gradient sedimentation, were fractionated as described before (37). The envelope fraction refers to material solubilized by treatment of virions with 50 mM dithiothreitol and 0.5% Nonidet P-40. All subsequent steps were performed at 4°C. The virus core was extracted with buffer containing 0.1% sodium deoxycholate (DOC) in 0.3 M Tris-HCl (pH 8.0)–50 mM dithiothreitol–0.25 M NaCl. Material solubilized by this treatment is referred to as the DOC-1 fraction. The 0.1% DOC-insoluble material was reextracted with 0.2% DOC in buffer A (37). Protein rendered soluble by 0.2% DOC (DOC-2 fraction) was separated by centrifugation from the residual insoluble material (pellet fraction). Soluble protein fractions were freed of nucleic acid by DEAE-cellulose chromatography as described before (37). The DEAE-I fractions were applied to a second DEAE-cellulose column at 70 mM NaCl; bound material (DEAE-II fraction, containing vaccinia virus RNA polymerase) was eluted with 0.25 M NaCl (37). RNA polymerase and VETF were purified by sedimentation of the DEAE-II fraction through an 11-ml 15 to 30% glycerol gradient containing 0.2 M NaCl in buffer A. Gradients were centrifuged at 41,000 rpm for 23 h in an SW41 rotor. Fractions (0.45 ml) were collected from the bottom of the tube. Nonspecific RNA polymerase activity was assayed as nucleotide monophosphate incorporation into acid-insoluble material in the presence of single-stranded DNA template and manganese (39). VETF promoter-binding activity was assayed as described before (6). Capping enzyme was assayed by enzyme-GMP complex formation (37, 38).

Transcription in vitro. Reaction mixtures (25 μl) containing 20 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM ATP, 1 mM UTP, 10 μM [α-^{32}P]CTP, 300 ng of linear DNA template (*Sma*I-cut pSB24 containing a vaccinia virus early promoter fused to a 390-nucleotide G-less cassette [22]), RNA polymerase, and VETF (as indicated) were incubated for 60 min at 30°C. Labeled transcription products were recovered and analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) as described before (19). Labeled RNAs were visualized by autoradiography of dried gels; transcription activity was quantitated by excising gel slices containing the 390-nucleotide runoff transcript and counting radioactivity in a liquid scintillation counter.

RESULTS

Growth characteristics of mutant viruses. Stocks of mutant virus prepared at the permissive temperature (31°C) were tested for their ability to form plaques at both 31 and 40°C in BSC40 cells. WT virus was also assayed. The efficiency of plaque formation by WT virus was similar at 31 and at 40°C. In contrast, all *ts* mutants formed plaques poorly at 40°C (Table 1). The number of plaques was reduced by >1,000-fold at the nonpermissive temperature in each case. The appearance of microplaques in the titration of *ts*55 suggested a leakiness in this mutant that allowed slow growth at the nonpermissive temperature. Condit et al. (10), in their original description of the *ts* mutants, had noted *ts*55 to be extremely leaky.

In order to determine whether defective plaque formation was due to a failure of *ts* virus to replicate or a failure to spread, one-step growth experiments were performed at both 31 and 40°C with all viruses. Cells were harvested at 2, 24, and 48 h after infection, and the production of infectious virus was assayed by titration at 31°C (Table 2). Growth of WT virus did not vary with temperature; the yield of WT progeny at 48 h (2×10^8 to 3×10^8 PFU) reflected a burst size of >100 PFU/cell. Growth of mutants *ts*1, *ts*31, and *ts*55 at the permissive temperature closely paralleled that of WT virus. At 40°C, *ts*1 and *ts*31 were wholly unable to produce infectious progeny above the residua of the inoculum reflected in the 2-h baseline titer. Thus, the plaque phenotype of these two mutants was clearly attributable to a profound thermosensitive defect in viral replication. Mutant *ts*55 did grow at 40°C, albeit much more slowly than at 31°C. This degree of leakiness was in keeping with the microplaque phenotype noted above. Mutant *ts*58 was exceptional in that its growth was impaired (relative to the WT and to the other *ts* mutants) even at the permissive temperature. Replication of *ts*58 at 40°C was defective relative to that at 31°C; an increase in titer above the background was detected only at 48 h.

TABLE 1. Thermosensitive plaque formation by mutant viruses[a]

Virus	Titer (PFU/ml)	
	31°C	40°C
WT	2×10^8	5×10^8
*ts*1	2×10^8	2×10^4
*ts*58	2×10^7	1×10^3
*ts*31	4×10^7	4×10^3
*ts*55[b]	5×10^7	$<1 \times 10^4$

[a] Virus in stocks was counted by serial 10-fold dilution onto BSC40 cells maintained at either 31 or 40°C. Plaques were visualized at 2 days postinfection by staining with 0.1% crystal violet in 20% ethanol.

[b] Numerous microplaques were noted on monolayers infected at 40°C with a 10^{-3} dilution of *ts*55 virus stock.

Analysis of viral protein synthesis by *ts* mutant viruses. The patterns of viral protein synthesis at the permissive and nonpermissive temperatures were analyzed by pulse-labeling synchronously infected cells with [^{35}S]methionine over a 12-h time course postinfection (Fig. 1). The normal developmental pattern of vaccinia virus gene expression was exemplified by cells infected with WT virus at 40°C, i.e., the appearance of novel early polypeptides at 2 to 4 h postinfection (visible against a background of host protein synthesis),

TABLE 2. One-step growth of mutant viruses at the permissive and nonpermissive temperatures[a]

Virus	Growth temp (°C)	Titer (PFU/ml)		
		2 h p.i.	24 h p.i.	48 h p.i.
WT	31	3×10^5	2×10^7	2×10^8
	40	4×10^4	3×10^7	3×10^8
*ts*1	31	2×10^5	3×10^7	5×10^7
	40	2×10^5	2×10^5	8×10^5
*ts*31	31	6×10^5	1×10^7	1×10^8
	40	6×10^5	3×10^5	6×10^5
*ts*55	31	5×10^5	1×10^7	7×10^7
	40	5×10^5	1×10^6	4×10^6
*ts*58	31	2×10^5	3×10^6	1×10^7
	40	1×10^5	1×10^5	1×10^6

[a] BSC40 cells maintained at 31 or 40°C were infected with WT or mutant virus at an MOI of 5. At the indicated times postinfection (p.i.), cells were harvested and counted by serial 10-fold dilution onto BSC40 cells at 31°C. Plaques were visualized at 2 days postinfection by staining with 0.1% crystal violet in 20% ethanol.

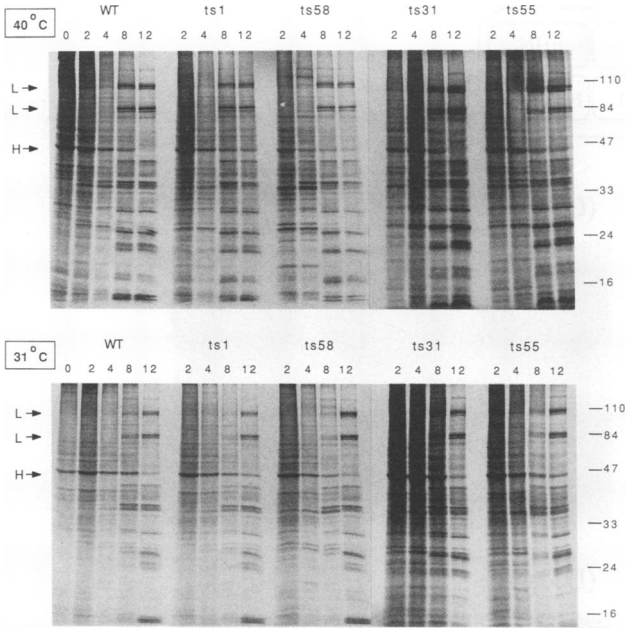

FIG. 1. Synthesis of viral proteins by *ts* mutant viruses. Virus-infected cells were pulse-labeled with [^{35}S]methionine, and radiolabeled polypeptides were analyzed by SDS-PAGE as described in Materials and Methods. Autoradiographs of the gels are shown. Cells were infected and maintained at either 40°C (top panel) or 31°C (bottom panel). The time at which pulse-labeling was initiated (expressed as hours postinfection) is indicated above each lane. Time 0 refers to uninfected cells. The identity of the infecting virus is noted above each set of time points. The positions and sizes (in kilodaltons) of coelectrophoresed protein standards are indicated at the right. Labeled polypeptides corresponding to the predominant 45-kDa host protein actin (H) and to two abundant viral late proteins (L) are indicated at the left by arrows.

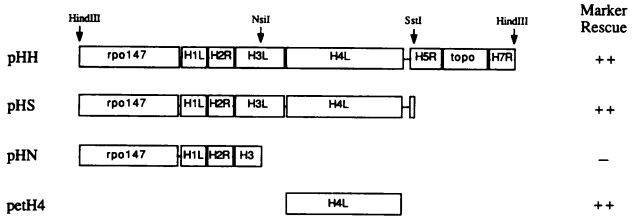

FIG. 2. Map of the genomic *Hin*dIII-H fragment and subfragments used for marker rescue. Plasmid pHH contains the full 8.6-kb *Hin*dIII-H fragment cloned into pUC13. A physical and genetic map of the viral DNA insert is shown. *Hin*dIII restriction sites defining the borders of the viral DNA and the internal sites for *Nsi*I and *Sst*I used in subcloning are indicated by vertical arrows. Protein-encoding regions of the H fragment (illustrated as boxed segments) are distinguished from noncoding regions (intervening line segments). Genes of known function are named accordingly (e.g., *rpo*147, encoding the largest subunit of RNA polymerase, and *topo*, encoding DNA topoisomerase I). Genes of unknown function are named according to the coventional poxvirus ORF notation (18, 34). The letters R (right) and L (left) in the ORF nomenclature refer to the direction of transcription of the gene. pHS and pHN contain subregions of the H fragment extending from the left *Hin*dIII site to either the *Sst*I site (pHS) or the *Nsi*I site (pHN). The genetic maps of these subregions are indicated in the figure. petH4 contains only the H4 ORF. A summary of the marker rescue results with each plasmid is shown at right.

transition to the synthesis of distinctive late proteins by 8 h (these include the major structural polypeptides p4a and p4b, indicated by L in Fig. 1), and shut-off of host protein synthesis late in infection (seen by loss of the prominent host actin polypeptide [H in Fig. 1]). Identical transitions were observed in cells infected with WT virus at 31°C; however, the timing of the appearance of viral proteins was delayed relative to that at 40°C. As anticipated, all four *ts* mutants manifested normal patterns of viral protein synthesis at the permissive temperature (Fig. 1, lower panel). Grossly normal protein synthesis patterns were also evident in cells infected with each mutant virus at the nonpermissive temperature (Fig. 1, upper panel). These cells had clearly progressed to the synthesis of the major late viral proteins (implying also that viral DNA replication had occurred). Thus, all four mutations within this complementation group displayed a normal phenotype. Condit et al. had reported previously that *ts*1 and *ts*55 were normal with respect to macromolecular synthesis (9). Closer inspection of the 12-h time point for *ts*58 at 40°C revealed the absence of a polypeptide of approximately 23 kDa that was otherwise present in WT-infected cells at 40°C as well as in *ts*58-infected cells at 31°C. The relevance of this finding to the thermolabile growth of this group of mutants is not clear.

Marker rescue analysis. It was shown previously that *ts*1 could be restored to temperature-independent growth by marker rescue with a plasmid containing the entire *Hin*dIII-H genomic fragment derived from WT virus (9, 10). (An annotated map of the 8.6-kb H fragment is illustrated in Fig. 2.) We have extended the analysis to all alleles of the complementation group by an electroporation-based protocol for DNA-mediated marker rescue (Fig. 3). In this method, cells infected with *ts* virus were electroporated with plasmid DNA (31) and plated onto uninfected cell monolayers maintained at the nonpermissive temperature. Successful marker rescue was expected to yield recombinant WT progeny from individual electroporated cells that were seeded onto the fresh monolayer; this was manifested as a plaque arising from each infectious center. No infectious centers were observed when monolayers were seeded with uninfected cells that had been electroporated without added DNA (Fig. 3, wells 6). Few or no infectious centers were seen with virus-infected cells that had been electroporated without added DNA (Fig. 3, wells 1).

Robust marker rescue was evident for each mutant when electroporation was performed with the pHH plasmid, containing the entire WT *Hin*dIII-H fragment (Fig. 3, wells 2). Comparable rescue was obtained with the H fragment subclone pHS (Fig. 3, wells 4). In contrast, only background plaque formation was observed when cells were electroporated with the more extensively deleted plasmid pHN (Fig. 3, wells 3). These results localized the *ts* mutations to a 3,063-bp segment between the *Nsi*I and *Sst*I restriction sites that included the entire H4L ORF, the amino-terminal half of the H3L ORF, and a short amino-terminal portion of the H5R ORF. Plasmid petH4, containing no vaccinia virus sequence extraneous to the H4L ORF, was able to rescue all mutant viruses with an efficiency equal to that of pHS or pHH (Fig. 3, wells 5). We concluded, therefore, that the genetic lesions of all four alleles occurred in the H4 gene.

Sequence analysis of the H4 gene. The 2,388-bp H4 gene of the Copenhagen strain of vaccinia virus (18) encodes a protein of 795 amino acids with a molecular mass of 93.6 kDa. Disparities in the ORF maps of the Copenhagen and

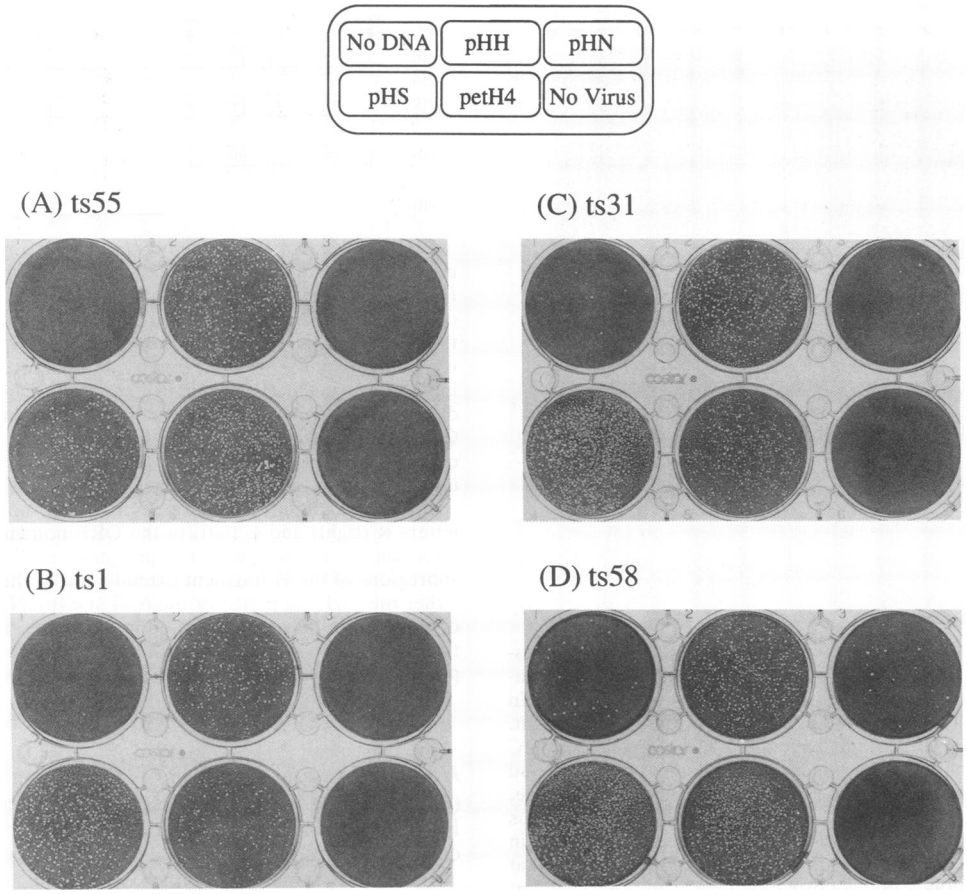

FIG. 3. Marker rescue of *ts* mutants. Electroporation-based marker rescue with supercoiled plasmid DNAs was performed for all four *ts* mutants as described in the text. Photographs of the stained monolayers are shown. Wells are numbered 1 to 6 (from top left to bottom right) on each culture dish. A key to the plasmids tested in each well of the marker rescue series is shown at the top. The "No Virus" control entailed seeding the monolayers with uninfected BSC40 cells that had been electroporated without added DNA.

WR strains of vaccinia virus in the H4L region (34) have been clarified by sequencing a newly constructed H fragment clone of WR genomic DNA. The wild-type WR H4L ORF, like that of the Copenhagen strain, encoded a polypeptide of 795 amino acids. The nucleotide sequence of the H4L (WR) ORF diverged from that of the H4L (Copenhagen) ORF at several positions, as indicated in Table 3. Base changes clustered at codons 623 and 624 resulted in alteration of the amino acid sequence from Cys-Val (Copenhagen) to Tyr-Ile (WR). Two other silent base changes were noted at codons 634 and 635 (Table 3).

The nucleotide sequences of the H4 genes of mutants *ts*1, *ts*55, and *ts*58 each diverged from that of WT H4 (Copenhagen) at a single position (Table 3). These base changes resulted in single amino acid substitutions within the H4 protein. The H4 gene of *ts*31 differed at two positions from the WT (Copenhagen) sequence, leading to amino acid substitutions at two distant sites in the polypeptide. Whether one or both of these point mutations are responsible for the thermolabile growth phenotype of *ts*31 remains to be determined. The sequence of the H4 gene of plasmid petH4, which rescued all *ts* alleles of the complementation group, encoded WT amino acids at all positions that were mutated in the *ts* isolates. A single missense mutation, Gln-424→Arg, was noted in petH4 relative to the WT (Copenhagen). The existence of this mutation (whether present in plasmid pHH or arising during PCR amplification of the H4 gene) does not alter our conclusions regarding the molecular mapping of the *ts* loci. The H4 nucleotide sequences of the *ts* WR mutants (isolated by Condit in 1981) were more highly related to the Copenhagen H4 sequence than they were to the WT WR H4 sequence (determined from genomic DNA isolated in 1991). This suggested that the WR strain propagated in our laboratory had incurred phenotypically silent base changes in the intervening years of passage.

Electron microscopy. Cells infected with WT or *ts*1 virus were harvested at 24 h postinfection and processed for electron microscopic examination. Mature brick-shaped virions were evident in the cytoplasm of *ts*1-infected cells maintained at either 31 or 40°C (Fig. 4B and D). The biconcave core and two flanking lateral bodies contained within a viral membrane were apparent in the high-magnification view shown in Fig. 4D. These particles were indistinguishable microscopically from those detected in cells infected with WT virus (Fig. 4A and C). The apparent heterogeneity of mature virions with respect to core condensation has been noted in prior microscopic analyses of vaccinia virus development (24).

The mature virion evolves in defined stages from well-characterized immature forms (8). Typical immature parti-

TABLE 3. Summary of DNA sequence changes in the H4 gene[a]

Virus	Base change(s)	Amino acid change(s)	Amino acid position(s)
*ts*1	CCA→CTA	Pro→Leu	716
*ts*55	GAA→AAA	Glu→Lys	357
*ts*58	GGA→AGA	Gly→Arg	367
*ts*31	AGT→AAT	Ser→Asn	745
	CCG→TCG	Pro→Ser	165
WT (WR)	TGT-GTA→TAC-ATC	Cys-Val→Tyr-Ile	623, 624
	TCT-GTA→TCG-GTC	Ser-Val (silent)	634, 635
petH4	CAA→CGA	Gln→Arg	424

[a] Nucleotide sequences were determined for the H4 coding region of genomic clones of the *Hin*dIII H fragment. Such clones were constructed with virion DNA from *ts* virus mutants and WT vaccinia virus WR. Also sequenced was the petH4 clone, used in the marker rescue analysis. All DNA base changes are reported relative to the published complete genomic sequence of vaccinia virus (Copenhagen) (18). In each case, the WT Copenhagen sequence is shown on the left of the arrow and that of the variant H4 gene is shown on the right. Affected nucleotides are underlined. Amino acid changes consequent to the base changes are also indicated. The positions of the affected amino acids within the protein sequence are indicated.

cles, with an intact spherical membrane enclosing diffuse nucleoplasm, were encountered in cells infected with *ts*1 at 40°C (Fig. 5B). Early membrane formation was seen, as were more mature structures, within which the nucleoplasm had begun to condense (Fig. 5B and C). Immature particles of *ts*1 were indistinguishable from those seen in cells infected with WT virus (Fig. 5C). We observed no aberrant viral structures unique to cells infected with *ts*1 at the nonpermissive temperature. We infer that the thermolability of the *ts*1 mutant was not caused by a gross qualitative defect in virus morphogenesis.

Localization of the H4 protein in virion cores. In order to examine whether the H4 protein is a virion component, antiserum prepared against an H4-specific synthetic peptide was used in Western blotting experiments. The polypeptide compositions of the virion envelope fraction, detergent extracts of virion cores (solubilized sequentially by 0.1 and 0.2% DOC), and the insoluble pellet fraction were examined by SDS-PAGE in order to monitor the integrity of the extraction procedure (Fig. 6, left panel). Virion proteins were partitioned in characteristic fashion; for example, the major virion polypeptides 4a and 4b were found exclusively in the insoluble pellet, as expected. Distinct subsets of viral proteins were released from cores by treatment with 0.1 and 0.2% DOC. Core-associated enzymes were among the proteins extracted differentially. For example, the vaccinia virus nucleotide triphosphatases were solubilized readily at 0.1% DOC, whereas RNA polymerase activity was recovered predominantly in the 0.2% DOC fraction (data not shown). The distribution of RNA polymerase was reflected in the abundance of the rpo147 and rpo135 subunits (indicated by the arrowhead in Fig. 6). A significant amount of the polymerase polypeptides (Fig. 6) and of RNA polymerase activity (data not shown) remained in the pellet fraction after extraction with detergents. Western blotting revealed an H4-immunoreactive polypeptide of 90 kDa present in the DOC extracts of virus cores but absent from the envelope fraction (Fig. 6, right panel). The size of this protein was consistent with that of the predicted H4 gene product. The H4 protein was more abundant in the 0.2% DOC extract than in the 0.1% DOC fraction. Residual H4 protein was also detected in the insoluble pellet fraction (data not shown). These data indicated that the H4 protein is a component of the vaccinia virus core.

Association of H4 protein with vaccinia virus RNA polymerase. Detergent-soluble core extracts were chromatographed through two DEAE-cellulose columns, first at high ionic strength (to remove nucleic acids) and then at low ionic strength. RNA polymerase activity is retained quantitatively during the DEAE-II step and recovered by elution with high salt (4, 39). The rpo147 and rpo135 polymerase subunits were evident in the DEAE-II fraction (Fig. 6, left panel). The immunoreactive H4 polypeptide was also retained on the DEAE-II column (Fig. 6, right panel). This fraction was purified further by sedimentation in a glycerol gradient. Nonspecific RNA polymerase activity (assayed with a single-stranded DNA template) sedimented as a discrete peak (S value, 15.5; data not shown) centered at gradient fraction 11 (Fig. 7, left panel). Virtually all of the immunoreactive 90-kDa H4 protein cosedimented with RNA polymerase; more specifically, the distribution of the H4 protein (peaking at fraction 10) was skewed toward the more rapidly sedimenting portion of the RNA polymerase activity peak (Fig. 7, center panel). The vaccinia virus capping enzyme, while capable of interacting with RNA polymerase in vitro at low ionic strength (19), was nonetheless clearly resolved from polymerase by this centrifugation step (Fig. 7, right panel). Capping enzyme activity (6.5 S) peaked in fraction 18; only a very small amount of guanylyltransferase cosedimented with polymerase. Similarly, VETF activity was encountered in gradient fractions 13 to 19 but not in fractions overlying the polymerase activity peak (data not shown). Thus, the H4 protein was associated with RNA polymerase under conditions that clearly resolved the polymerase from other components of the early transcription system.

Fractions across the polymerase activity peak were tested for their ability to transcribe an early promoter-containing duplex DNA template, pSB24 (22). Polymerase alone displayed extremely weak activity in specific transcription but could be stimulated markedly by the addition of VETF from less rapidly sedimenting fractions of the same gradient (Fig. 8, top panel). This result underscores the clear resolution of VETF from RNA polymerase during the centrifugation step. Specific transcription was quantitated by cutting out and counting the radioactivity present in the 390-nucleotide runoff transcript programmed by the pSB24 template (Fig. 8, bottom panel). It was apparent that the activity profile in specific transcription was skewed toward the more rapidly sedimenting portion of the polymerase activity peak. Whereas gradient fractions 10 and 12 displayed identical activity in nonspecific transcription (Fig. 7), fraction 10 had 2.7-fold more activity than fraction 12 in specific transcription. Indeed, the ratio of specific to nonspecific transcription activity declined progressively in going from "heavier" to "lighter" polymerase fractions.

The polypeptide composition of the more rapidly sedimenting side of the vaccinia virus polymerase peak from another glycerol gradient was analyzed by SDS-PAGE followed by silver staining (Fig. 9). Along with the expected subunits of the core vaccinia virus polymerase (4), we noted a prominent polypeptide of about 85 to 90 kDa (indicated by the arrowhead in Fig. 9). This species, which we presume represents the H4 protein, migrated with mobility similar to that of the sigma subunit of *E. coli* RNA polymerase. This polypeptide clearly did not correspond to the large subunit (96.7 kDa) of the vaccinia virus capping enzyme/termination factor (37), since its mobility was distinct from (and slightly faster than) that of capping enzyme/termination factor electrophoresed in parallel with the RNA polymerase fraction. Although not quantitated directly, the putative H4 polypep-

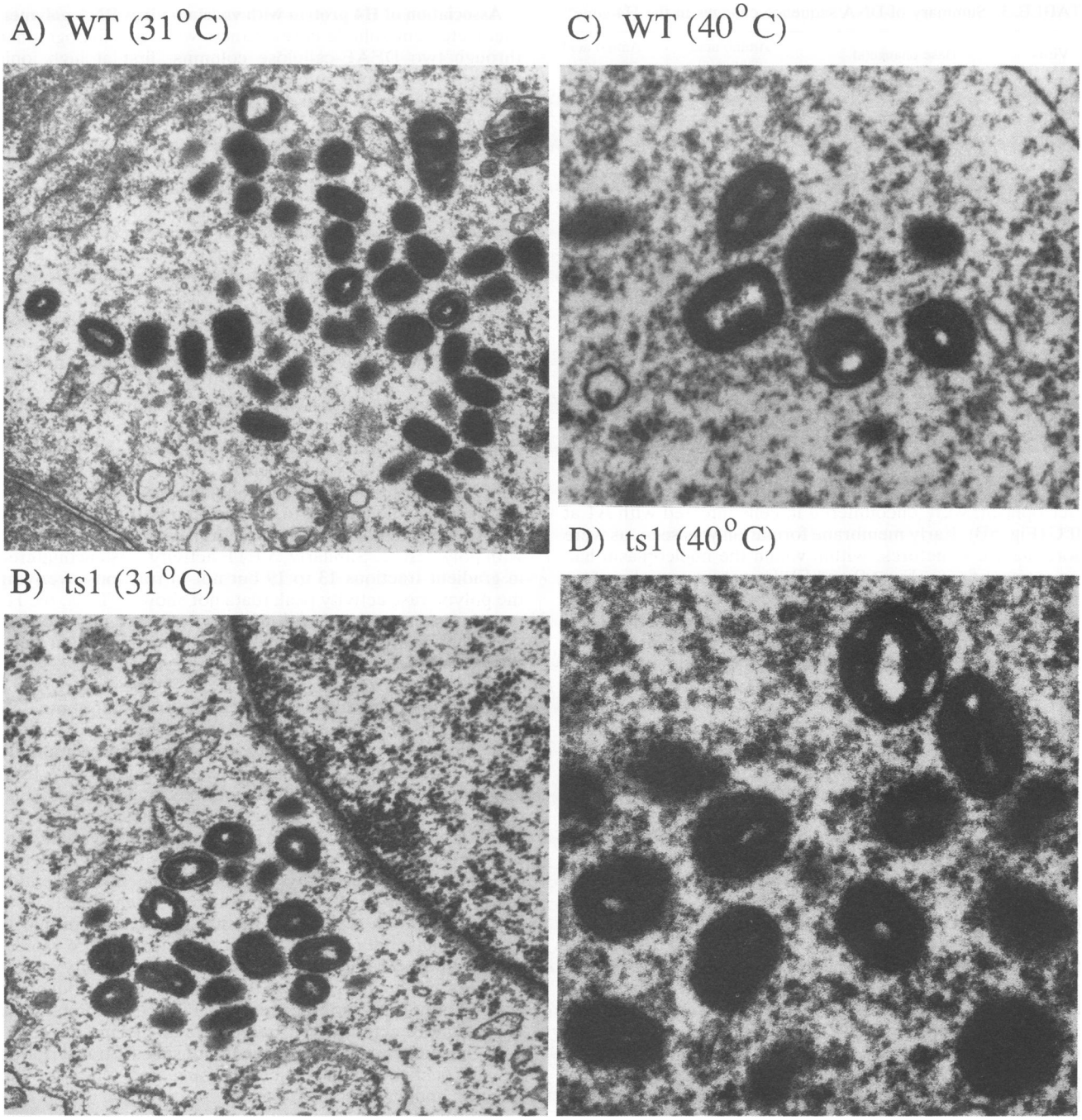

FIG. 4. Electron microscopic analysis of virus morphogenesis. Cells infected with WT or *ts*1 virus were harvested at 24 h postinfection and analyzed by electron microscopy. Representative micrographs of mature cytoplasmic virus particles are shown. Specimens were as follows (magnification in parentheses): (A) WT virus at 31°C (×10,000); (B) *ts*1 at 31°C (×12,000); (C) WT at 40°C (×20,000); (D) *ts*1 at 40°C (×25,000).

tide appeared to be present in nearly stoichiometric amounts relative to the rpo147 and rpo135 polymerase subunits. The data are consistent with the idea that the enhanced specific transcriptional activity of the heavy isoform of the polymerase is attributable to the presence of the H4 polypeptide as a component of polymerase "holoenzyme," distinct from and partially resolved during sedimentation from a transcriptionally less active or inactive "core" polymerase that lacks H4 protein. In keeping with this view, examination of a silver-stained gel of the gradient fractions in Fig. 7 revealed a 90-kDa species present in fractions 10 and 11 that declined in abundance (relative to rpo147 and rpo135) in fractions 12 and 13 (data not shown). Our studies do not exclude the possibility that cosedimentation of H4 with polymerase is coincidental (e.g., because native H4 protein is a hexamer), although we consider this an unlikely scenario.

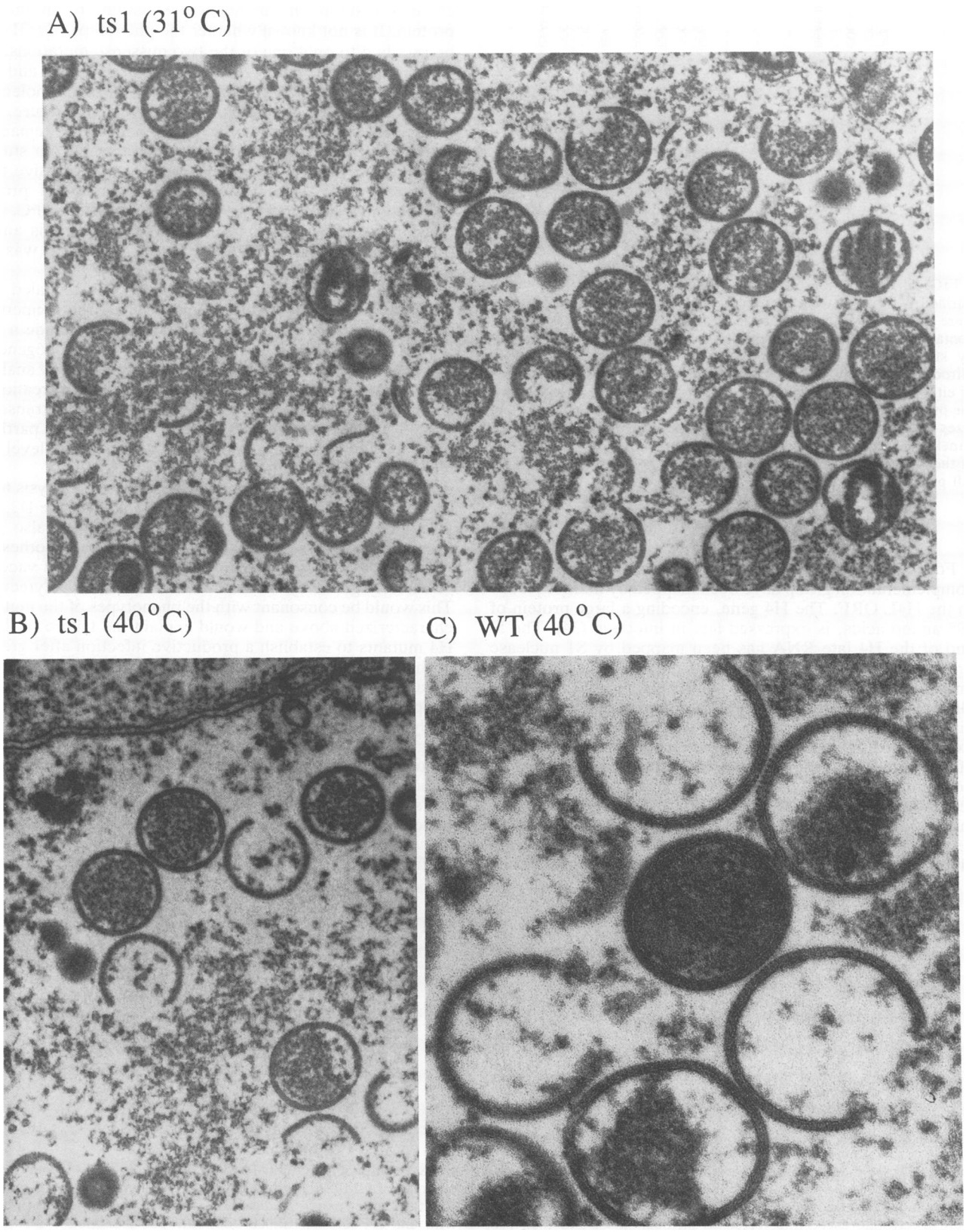

FIG. 5. Microscopic examination of immature viral particles. Cells infected with WT or *ts*1 virus were harvested at 24 h postinfection and analyzed by electron microscopy. Representative micrographs of immature cytoplasmic virus particles are shown. Specimens were as follows (magnification in parentheses): (A) *ts*1 at 31°C (×20,000); (B) *ts*1 at 40°C (×20,000); (C) WT at 40°C (×40,000).

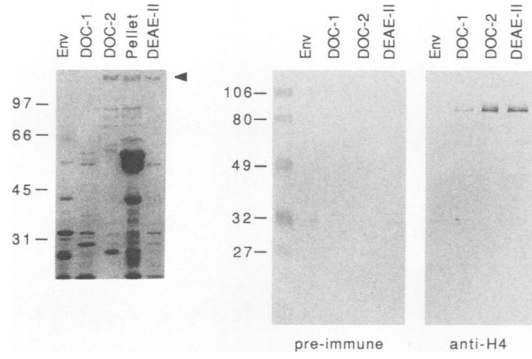

FIG. 6. Association of H4 protein with virus cores. Extracts of purified WT virions were prepared as described in the text. Aliquots were analyzed by electrophoresis through a 10% polyacrylamide gel containing 0.1% SDS. Polypeptides were either visualized directly by staining with Coomassie Blue (left panel) or transferred to nitrocellulose membranes for immunoblotting with 1:2,000 dilutions of either preimmune or anti-H4 serum (right panel). The source of the protein fraction is indicated above each lane. The locations and sizes (in kilodaltons) of marker proteins are shown at the left of each panel. The doublet corresponding to the rpo147 and rpo135 subunits of the virion RNA polymerase is indicated by an arrowhead in the left panel.

DISCUSSION

Four vaccinia virus *ts* mutations from a single genetic complementation group have been mapped by marker rescue to the H4L ORF. The H4 gene, encoding a large protein of 795 amino acids, is expressed late in infection (34). The 5′ end of the H4 late RNA has been mapped by S1 nuclease protection to within a few bases of the sequence TAAATG immediately preceding the translational start site of the H4 ORF (34). This motif is essential for the function of vaccinia virus late promoters (12, 25). Also consistent with the expression of H4 at late times is the presence within the ORF of two copies of the TTTTTNT early transcription termination signal (42), an element that is excluded from the coding regions of most viral early genes. The H4 gene of each mutant virus was sequenced to localize the genetic lesion responsible for the *ts* phenotype. Each virus had sustained either one (*ts*1, *ts*55, and *ts*58) or two (*ts*31) nucleotide changes resulting in amino acid substitutions in the H4 protein. It is not known whether the phenotype of *ts*31 may be ascribed to only one of the two missense mutations.

Condit and Motyczka (9) had already assigned *ts*1 and *ts*55 to the class of mutants displaying normal macromolecular synthetic patterns at the nonpermissive temperature. We have extended this phenotypic assignment to the remaining members of the complementation group through our studies of protein synthesis at permissive and nonpermissive temperatures. A WT profile of early and late viral protein production was evinced by the *ts* mutants at 40°C. The possibility that subtle changes in protein synthesis and/or processing might contribute to the *ts* phenotype was not excluded by our experiments.

Electron microscopy of *ts*1-infected cells revealed that assembly of progeny virions could occur at the nonpermissive temperature. This does not completely exclude a role for the H4 gene product in vaccinia virus morphogenesis; morphogenetic phenotypes might yet be revealed by analysis of other mutations in the H4 gene or through the creation of a "null" H4 gene by conditional repression of its transcription (33, 43). It is likely that defects exist in *ts*1 particles made at 40°C which are not detectable at a gross level and which render the virions noninfectious.

The H4 protein was shown by Western blot analysis to be encapsidated within the virus core particle (like the D2, D3, D6, and I8 gene products, mutations in which also give normal phenotypes [16, 17, 36]). An attractive hypothesis is that the H4 protein is required for transcription of vaccinia virus early genes by the core-associated RNA polymerase. This would be consonant with the phenotypes of the mutants characterized above and would explain the failure of the *ts* H4 mutants to establish a productive infection after growth at the higher temperature. We are addressing this issue by isolating the virus particles produced at the nonpermissive temperature and subjecting them to structural and functional analysis. Preliminary studies of the *ts*1 mutant indicate that progeny virions made at 40°C are indistinguishable from particles made at the permissive temperature with respect to sedimentation properties and composition of the major virion structural proteins. However, the virions made at 40°C were >1,000-fold less infectious than virions made at 31°C (based on PFU/A_{260} of sucrose gradient-purified virus preparations).

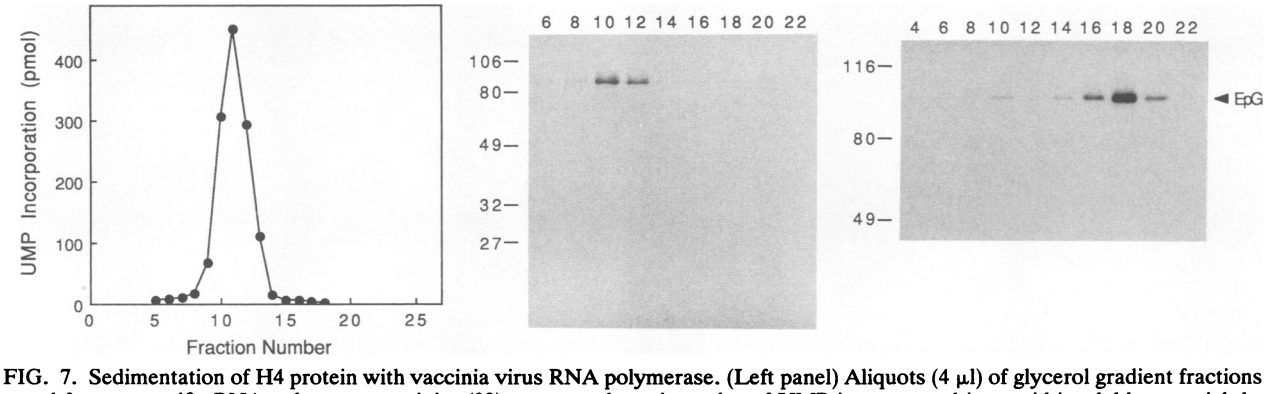

FIG. 7. Sedimentation of H4 protein with vaccinia virus RNA polymerase. (Left panel) Aliquots (4 μl) of glycerol gradient fractions were assayed for nonspecific RNA polymerase activity (39), expressed as picomoles of UMP incorporated into acid-insoluble material during a 30-min incubation at 37°C. The direction of sedimentation is from right to left. (Center panel) Aliquots (20 μl) of glycerol gradient fractions were electrophoresed, transferred to nitrocellulose, and then probed with anti-H4 serum (1:2,000 dilution). The locations and sizes (in kilodaltons) of marker proteins are indicated at the left. (Right panel) Aliquots (1 μl) of glycerol gradient fractions were assayed for guanylyltransferase activity by formation of radiolabeled nucleotidyl-enzyme complex (EpG). Covalently labeled 95-kDa capping enzyme subunit (38) was identified by autoradiography after SDS-PAGE analysis of reaction mixtures. The locations and sizes (in kilodaltons) of marker proteins are noted at the left. The location of EpG is indicated by the arrowhead at right.

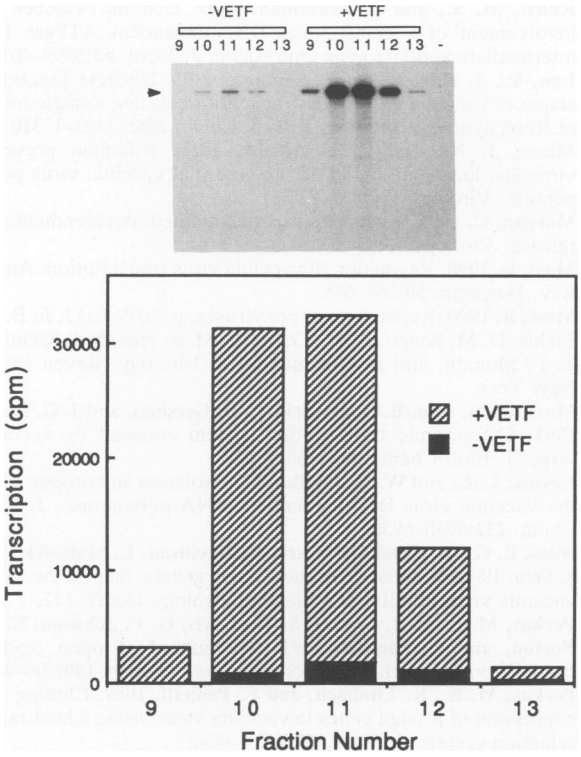

FIG. 8. Activity of glycerol gradient polymerase fractions in early transcription. (Top panel) Transcription reaction mixes were programmed with pSB24 template and contained the indicated polymerase fractions of the glycerol gradient (from Fig. 7) plus or minus VETF. A control reaction mix containing only VETF was included (lane —). Radiolabeled RNA products were resolved electrophoretically and visualized by autoradiography. The position of the major runoff transcript is indicated by the arrow. (Bottom panel) Transcription activity with and without VETF was quantitated by scintillation counting of gel slices containing the runoff RNA.

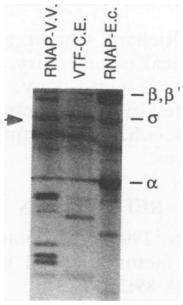

FIG. 9. Polypeptide composition of RNA polymerase sedimented in a glycerol gradient. Sedimentation of the DEAE-II polymerase fraction through a 15 to 35% glycerol gradient was performed as described before (37). An aliquot (30 μl; 1.2 U of RNA polymerase activity) of a fraction corresponding to the more rapidly sedimenting side of the vaccinia virus polymerase peak was electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS (lane RNAP-V.V.). Electrophoresed in parallel (lane VTF-C.E.) was a sample of the vaccinia virus capping enzyme/transcription termination factor (glycerol gradient fraction). *E. coli* RNA polymerase was coelectrophoresed in lane RNAP-E.c. A photograph of a silver-stained gel is shown. The subunits of the bacterial polymerase are indicated at the right. The location of a polypeptide in the vaccinia virus RNA polymerase presumed to correspond to the H4 protein is indicated by the arrowhead at the left.

The finding that the wild-type H4 polypeptide is associated physically with the vaccinia virus RNA polymerase during purification is consistent with a role for H4 in transcription. Indeed, H4 protein is associated with the more rapidly sedimenting population of polymerase molecules that are most active in VETF-dependent transcription of early genes in vitro. The abundance of the H4 protein in the transcriptionally active polymerase preparation suggests that H4 might be a true subunit of a holoenzyme form of polymerase.

The H4 protein is clearly not required in stoichiometric amounts for nonspecific RNA polymerase activity on single-stranded DNA templates, insofar as the most highly purified RNA polymerase fractions obtained from virions contain no polypeptide corresponding to the H4 protein in size (4). However, inspection of the published polypeptide compositions of less exhaustively purified preparations of virion polymerase (including preparations isolated by glycerol gradient sedimentation) reveals variable amounts of an approximately 85- to 90-kDa species (2, 7, 32). It has also been observed that an approximately 90-kDa radiolabeled polypeptide is recovered in immunocomplexes precipitated from infected cells by antiserum directed against the rpo19 subunit of vaccinia virus RNA polymerase (2). Sequencing of tryptic peptides derived from the 85-kDa polypeptide associated with virion RNA polymerase showed recently that this species corresponds to the H4 gene product (1). Ahn and Moss observed that H4 protein is associated with an isoform of polymerase that is separable from core polymerase lacking this protein and that specific transcription of early genes in vitro in the presence of VETF is confined to the H4-containing form of polymerase (1). Transient-transfection experiments provide strong evidence that H4 protein is indeed required for early transcription (1).

Vaccinia virus RNA polymerase purified to apparent homogeneity from the soluble cytoplasmic fraction of late-infected HeLa cells contains stoichiometric amounts of a putative subunit of 77 kDa, as estimated by SDS-PAGE (28). We suspect that this subunit, identified by Nevins and Joklik (28), corresponds to the H4 protein. It is likely that in purifying the polymerase from the soluble fraction (rather than from the insoluble cytoplasmic fraction, containing newly forming virions and subviral particles), Nevins and Joklik obtained a preparation containing exclusively the holoenzyme. Insofar as extraction of virions entails treatment with ionic detergents, the polymerase solubilized from cores may well be heterogeneous with respect to content of polypeptides dispensable for nonspecific RNA synthesis but perhaps required for specific transcription. Indeed, we have found that even after extraction with 0.2% DOC, considerable amounts of the RNA polymerase large subunits and of the H4 protein remain associated with the insoluble pellet. Thus, the stoichiometry of H4 protein in the extracted polymerase fraction may not reflect the molar ratio within the core particle.

Because the core RNA polymerase subunits and the capping enzyme subunits are all synthesized early in infection (25, 27), whereas the H4 gene is expressed at late times (1, 34), it is unlikely that the form of RNA polymerase that transcribes intermediate and late genes would require or contain H4 protein as an integral holoenzyme subunit. Thus, cascade models proposed for vaccinia virus gene regulation by accessory transcription factors (5, 25, 27) may also apply to stage-specific components of the polymerase holoenzyme.

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