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Genetic Evidence for Involvement of Vaccinia Virus DNA-Dependent ATPase I in Intermediate and Late Gene Expression

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To delineate the role of the vaccinia virus-encapsidated DNA-dependent ATPase I in the life cycle of the virus, we performed a detailed study of two temperature-sensitive mutants with lesions in the gene encoding the enzyme. Profiles of viral DNA and protein accumulation during infection showed the mutants to be competent for DNA synthesis but deficient in late protein synthesis, confirming their defective late phenotype (R. C. Condit and A. Motyczka, Virology 113:224–241, 1981; R. C. Condit, A. Motyczka, and G. Spizz, Virology 128:429–443, 1983). In vitro translation of viral RNA and S1 nuclease mapping of selected mRNAs demonstrated that the deficit in late protein synthesis stemmed from a defect in the transcriptional machinery. Intermediate and late gene expression appeared to be most affected. The transcriptional defect was of unequal severity in the two mutants. However, their phenotypes were indistinguishable and their respective lesions were mapped to the same 300 nucleotides at the 5′ end of the gene. DNA sequence analysis assigned a single nucleotide and amino acid change to one of the mutants.

The replication and transcription of DNA involve complex interactions between *cis*-acting sites, binding proteins, and enzymes. Beyond accomplishing specific and accurate initiation and elongation of RNA and DNA chains, this machinery must also provide for the partial unwinding of the DNA helix and the resolution of the torsional stress which accumulates during replication and transcription. Productive study of these processes is aided by the availability of experimental systems amenable to both biochemical and genetic analyses. Vaccinia virus, a highly autonomous virus which replicates lytically in the cytoplasm of infected cells, provides these experimental advantages. The virus, which possesses a linear DNA genome of 185 kilobases (kb) and whose life cycle involves an orderly progression through temporally regulated phases of early gene expression, DNA replication, and late gene expression, is thought to encode all of the functions required for DNA metabolism (for a review, see reference 22). Among the virus-specific enzymes detected in virions or infected cells are DNA polymerase, thymidine kinase, ribonucleotide reductase, topoisomerase, nucleases, RNA polymerase, guanylyl transferase, poly(A) polymerase, transcription factors, transcriptional initiation and termination factors, and two DNA-dependent ATPases (for a review, see reference 22). The genes for many of these proteins have been identified, including that for one of the DNA-dependent ATPases (5, 25, 30). DNA-dependent ATPases have been identified in a number of biological systems and been found to play important roles in transcription, replication, and recombination (10, 13, 17, 19, 23, 34, 36). Most known DNA helicases are DNA-dependent ATPases (15, 20, 33, 37), as are several transcription factors (3, 35) and recombination proteins such as RecA and Rec-1 (9).

The vaccinia virus-encapsidated DNA-dependent ATPase I has been purified and analyzed (5, 26, 27). Our investigations have confirmed that the enzyme is a monomer of 68 kilodaltons (kDa) which will hydrolyze ATP or dATP in the presence of a DNA cofactor. Our efforts to detect other enzymatic activities, such as helicase activity, have been unsuccessful. Therefore, to further understand the biochemical properties and physiological role of the ATPase, we have turned to the analysis of a complementation group of vaccinia virus mutants which have lesions within the gene encoding the DNA-dependent ATPase I. The preliminary characterization of these temperature-sensitive (*ts*) mutants identified them as defective late mutants which exhibited a striking defect in the synthesis of the late class of viral proteins during infection at the nonpermissive temperature (7, 8). In this report, we present analyses of the patterns of DNA replication, protein synthesis, and RNA synthesis during infection with these mutants at the permissive and nonpermissive temperatures. Moreover, we describe the fine mapping of the lesions within these mutants and the identification of a single nucleotide change responsible for the conditionally lethal phenotype of one of the mutants.

MATERIALS AND METHODS

Materials. Restriction endonucleases were obtained from New England BioLabs, Inc. (Beverly, Mass.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used according to the instructions of the manufacturers. Polynucleotide kinase, calf intestinal phosphatase, S1 nuclease, *Escherichia coli* DNA polymerase I, the Klenow fragment of *E. coli* DNA polymerase I, T4 DNA ligase, and pancreatic RNase were from Boehringer Mannheim; ^{32}P-labeled nucleotide triphosphates and [^{35}S]methionine were obtained from New England Nuclear Corp. (Boston, Mass.). ^{14}C-labeled high-molecular-weight standards were from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

Cells and virus. Wild-type (wt) vaccinia virus, WR strain, was provided by Bryan Roberts. The *ts* mutants *ts*36 and *ts*50 were provided by Richard Condit (7, 8). In the virological studies of these mutants, 32.0 and 39.5°C were used as the permissive and nonpermissive temperatures, respectively. Stocks of viral mutants were grown at the permissive temperature on monolayers of mouse L cells, while wt virus was propagated in suspension cultures of L cells. Mouse L cells and African green monkey cells (BSC-40) were obtained from R. Condit and maintained in Dulbecco minimal

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essential medium (DMEM) with 5% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). L cells for suspension cultures were provided by J. Kates and maintained in Joklik modified essential medium with 2.5% calf serum–2.5% horse serum (GIBCO). BSC-40 cells were used for viral plaque assays.

Southern dot blot analysis. Confluent L-cell monolayers were infected with wt or mutant virus at a multiplicity of infection (MOI) of 15. After adsorption with virus for 30 min in DMEM, the inoculum was removed and the cells were fed with DMEM–5% fetal bovine serum. At various times postinfection, cells were scraped from the dish and washed with phosphate-buffered saline. After collection by centrifugation, cell pellets were suspended in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1 M ammonium acetate, freeze-thawed three times, and then diluted with 3 volumes of 10× SSC–1 M ammonium acetate. Lysates were stored at −20°C. Southern DNA analysis was performed using a Zeta-Probe blotting membrane and a dot blot apparatus from Bio-Rad Laboratories (Richmond, Calif.). As previously described (12), the membrane was prewetted in water and washed with 10× SSC–1 M ammonium acetate prior to loading the samples of lysate representing 2×10^4 cells. DNA was denatured in situ with 0.5 M NaOH–1.5 M NaCl; the filter was neutralized with two washes of 10× SSC. The membrane was air dried and baked for 30 min at 80°C in vacuo. Southern hybridization was carried out in 6× SSC –50% formamide –1× Denhardt solution (50× Denhardt solution is 10 mg of bovine serum albumin per ml, 10 mg of Ficoll per ml, and 10 mg of polyvinylpyrrolidone per ml) by standard procedures. The probe used was a molecular clone of the wt vaccinia virus *Hin*dIII D fragment radiolabeled by nick translation (29). Blots were examined by autoradiography. Viral DNA accumulation during the course of infection was then quantitated: each dot on the filter was cut out and the amount of bound probe was measured by Cerenkov counting in a Packard scintillation counter. Counts per minute were plotted for each sample against time postinfection.

Metabolic labeling. Confluent monolayers of L cells were infected with wt or mutant virus at an MOI of 15. Virus was adsorbed for 30 min in DMEM at the indicated temperature. The inoculum was then removed, and the cells were rinsed and fed with DMEM–5% fetal bovine serum. At various times postinfection, cultures were washed with methionine-free DMEM and incubated for 30 min in prewarmed methionine-free DMEM supplemented with [^{35}S]methionine at 100 μCi/ml. The monolayers were then washed with phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.4]) and lysed with ice-cold PLB (0.1 M $NaPO_4$ [pH 7.4], 0.1 M NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate). The lysates were stored at −20°C until analysis by SDS-polyacrylamide gel electrophoresis (PAGE) by standard procedures (18). Gels were examined by autoradiography.

RNA isolation and in vitro translation. L-cell monolayers were infected with the indicated virus at an MOI of 15. Infection and incubation of the monolayers were carried out as described above. At various times postinfection, cells were washed with phosphate-buffered saline and lysed with guanidinium thiocyanate (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% lauroylsarcosine, 0.1 M β-mercaptoethanol). The resulting lysates were passed four times through a 23-gauge needle, and the RNA was isolated by centrifugation through a cesium chloride cushion (5.7 M CsCl, 0.1 M EDTA [pH 7.0]). Total RNA was collected, suspended in water, and stored at −80°C. Aliquots of RNA from each time point were translated in a message-dependent rabbit reticulocyte lysate (28) (Green Hectares, Oregon, Wisc.) supplemented with 2 μCi of [^{35}S]methionine per μl. After incubation for 30 min at 37°C, translation was stopped by digestion of RNA for 15 min with excess pancreatic RNase. Translation products were analysed by SDS-PAGE, and gels were examined by autoradiography.

S1 nuclease analysis. Probes were labeled at the 5′ terminus with [γ-^{32}P]ATP by standard procedures. Probes used to detect mRNAs from the vaccinia virus I_3 (32, 43) and 11K protein (2, 45) genes were obtained from plasmids provided by H. Stunnenberg. Note that I_3 refers to the Vos-Stunnenberg nomenclature (43) of the open reading frames in the *Hin*dIII I genomic fragment and corresponds to open reading frame I_2 in the nomenclature of Tengelsen et al. (38), in which I_3 is the gene encoding the large subunit of ribonucleotide reductase. Each probe was suspended in 80% formamide–1× HARTS [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 400 mM NaCl, 1 mM EDTA], in the presence or absence of RNA (isolated as described above), under conditions of DNA excess. Nucleic acids were heat denatured at 68°C and allowed to rehybridize for 3 h at 38°C in a volume of 30 μl. The samples were then diluted with 300 μl of ice-cold S1 nuclease buffer (0.28 M NaCl, 0.05 M sodium acetate [pH 4.6], 0.5 mM $ZnSO_4$) containing sonicated salmon sperm DNA at 20 μg/ml; S1 nuclease was at a concentration of 400 U/μl when added. After 30 min of incubation at 45°C the samples were extracted twice with organic solvent, precipitated with ethanol, and analyzed by electrophoresis on thin 4% urea–polyacrylamide gels. The gels were examined by autoradiography.

DNA preparation and molecular cloning. The wt *Hin*dIII D clone was provided by B. Moss. Subfragments of wt *Hin*dIII-D obtained by restriction enzyme digestion were cloned in pBR322, pUC-8, or pKK223-3. Genomic DNA from *ts*36 and *ts*50 was obtained by harvesting monolayers of infected cells at the point of maximal cytopathic effect. The cells were lysed by hypotonic swelling, the nuclei were removed by centrifugation, and the viral cores were then collected by centrifugation. The cores were treated by digestion with proteinase K plus SDS, and the viral DNA was extracted with phenol-chloroform(isoamyl) and chloroform(isoamyl) and precipitated by ethanol. DNA fragments were purified from agarose by glass powder purification (42). The *ts*36 and *ts*50 *Hin*dIII D fragments and their subfragments were cloned in pBR322 or pUC-8. Restriction enzyme digestions, additions of oligonucleotide linkers to DNA fragments, and molecular cloning were done by standard procedures. Plasmids were grown under appropriate antibiotic selection in the HB101 or K440 strain of *E. coli* and purified by the alkaline lysis procedure (16).

Marker rescue. Confluent monolayers of BSC-40 cells were infected with *ts* mutant virus at an MOI of 0.03 and maintained at the permissive temperature for 4 h. Ten micrograms of linearized wt plasmid DNA was then added in the form of a calcium phosphate precipitate (24, 40), and the cells were shifted to the nonpermissive temperature. After 3 days at 39.5°C, the cells and media were harvested, subjected to two cycles of freeze-thawing, and treated with two 15-s bursts of sonication. Serial dilutions of the preparations were analyzed by plaque assays performed at both the permissive and nonpermissive temperatures. After 48 h of incubation, the assay plates were fixed and stained with 3.7% formaldehyde–0.1% crystal violet.

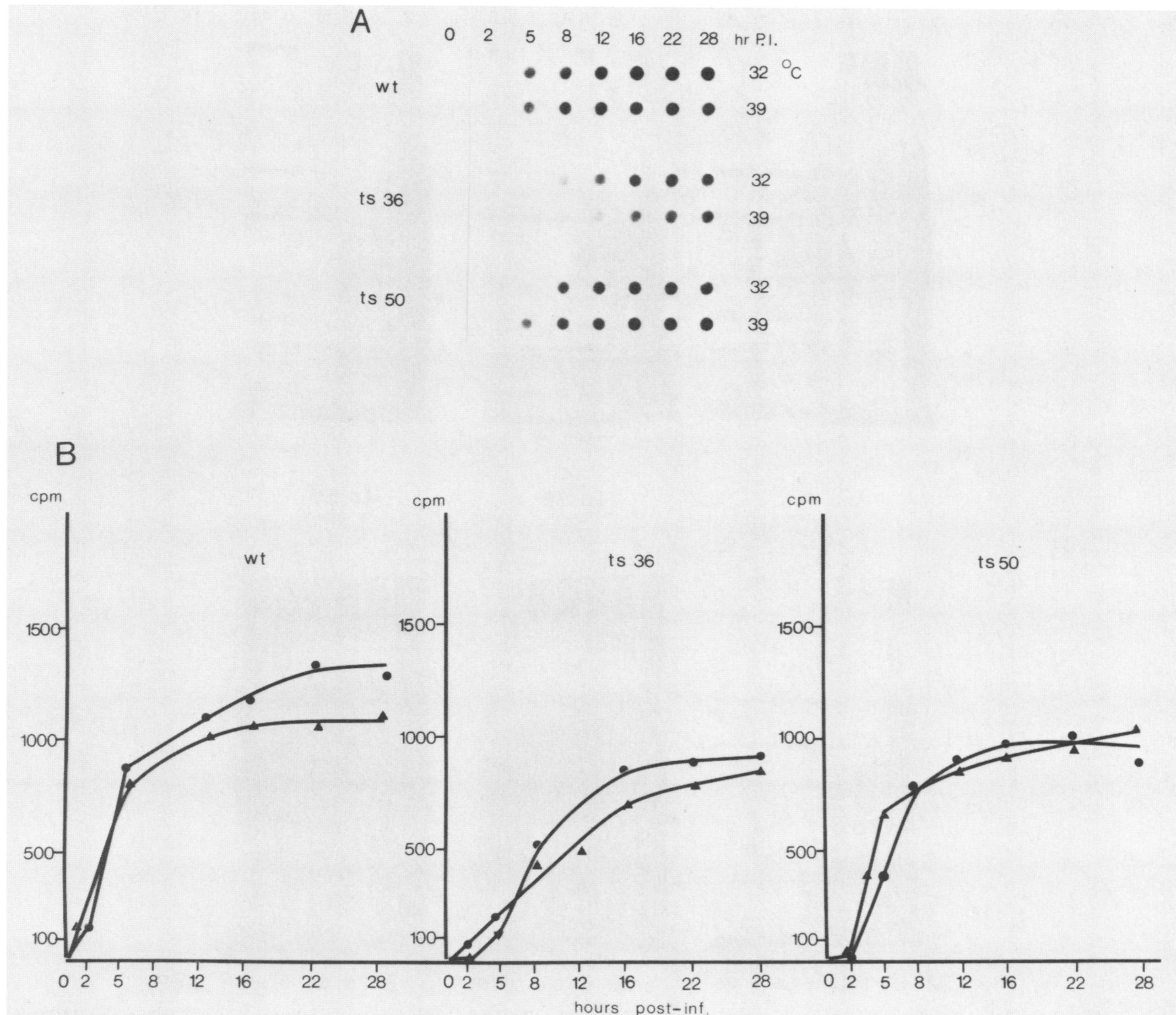

FIG. 1. Southern dot blot analysis of viral DNA accumulation in wt- and mutant-infected cells. L cells were infected with wt, *ts*36, or *ts*50 virus (MOI of 15) and incubated at 32 or 39.5°C. At the indicated times postinfection (P.I.), the cells were lysed and an aliquot of each lysate was immobilized on a Zeta-Probe membrane (nylon). At 0 h postinfection, lysates were prepared from mock-infected cells. DNA was denatured in situ and probed with a ^{32}P-labeled 16-kb *Hin*dIII D vaccinia virus genomic fragment. (A) Autoradiograph of the membrane. (B) Quantitation of viral DNA accumulated during the course of infection. Symbols: ●, 32°C; ▲, 39°C. The amount of probe hybridizing to each sample was determined by Cerenkov counting.

DNA sequencing. Plasmid DNA was digested with the appropriate restriction enzymes and labeled either at the 3′ terminus with the Klenow fragment of DNA polymerase I and the appropriate α-^{32}P-deoxynucleoside triphosphate or at the 5′ terminus with polynucleotide kinase and [α^{32}P]ATP after dephosphorylation with calf intestinal phosphatase. Radiolabeled DNA was then subdigested to yield a DNA fragment labeled at a single end. Fragments were purified from agarose by glass powder purification and sequenced by the Maxam-Gilbert chemical degradation procedure (21). Sequences were obtained for both strands and analyzed on an IBM PC with the IBI DNA sequence software.

RESULTS

Viral DNA synthesis during wt and mutant virus infection. A preliminary characterization of the vaccinia virus mutants *ts*36 and *ts*50 (7, 8) had indicated that the two mutants were competent for DNA replication and deficient in late protein synthesis. In these previous studies, [^{3}H]thymidine incorporation was used as an indicator of viral DNA synthesis. Such an approach, however, is not able to detect defects in virus-specific DNA accumulation which may occur later than 5 h postinfection. For this reason, we chose Southern dot blot analysis to monitor and quantitate the accumulation of viral DNA during a 28-h course of infection (Fig. 1A). The kinetics of viral DNA accumulation in wt-, *ts*36-, and *ts*50-infected cells are shown in Fig. 1B. Aliquots of total-cell lysates prepared at 2, 5, 8, 12, 16, 22, and 28 h after infection were immobilized on a nylon membrane; viral DNA was hybridized to an excess of radiolabeled DNA representing the 16-kb *Hin*dIII D vaccinia virus genomic fragment. A plot of viral DNA accumulation is shown in Fig. 1B.

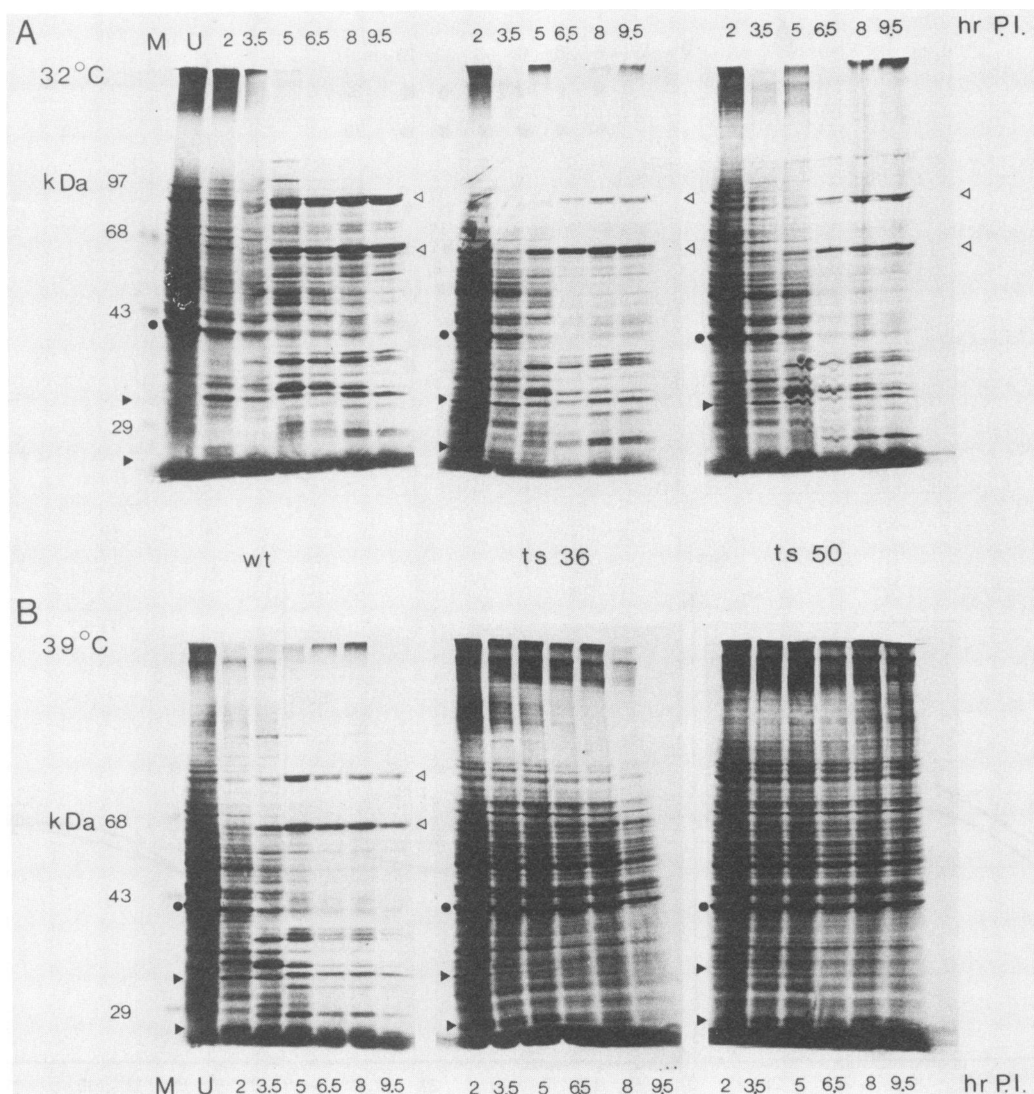

FIG. 2. Metabolic labeling of viral proteins in wt- and mutant-infected cells. Cells were infected with wt, *ts*36, or *ts*50 virus (MOI of 15) and incubated at 32 or 39.5°C. At various times postinfection (PI), cells were pulsed with [^{35}S]methionine for 30 min and lysed at the indicated times. Labeled proteins were separated by SDS-PAGE. Autoradiographs of the gels are shown. In both panels, lane M shows the migration of ^{14}C-labeled protein standards and lane U represents a lysate prepared from mock-infected cells. Cellular actin (●), abundant early viral proteins (▶), and abundant late viral proteins (◁) are indicated.

The results confirm that both *ts*36 and *ts*50 are capable of replicating their DNA at the nonpermissive temperature (39.5°C). The levels of virus-specific DNA accumulated during infection with either mutant at the nonpermissive temperature were indeed comparable to those reached at the permissive temperature. The appearance of detectable virus-specific DNA sequences was, however, delayed by approximately 3 h in *ts*36-infected cells, both at the permissive and at the nonpermissive temperatures. Moreover, the level of DNA accumulation achieved by either mutant at either temperature was reduced compared with that seen in wt virus infection. In spite of these minor defects, neither *ts*36 nor *ts*50 displayed a temperature-sensitive phenotype for DNA replication.

Analysis of viral protein synthesis by metabolic labeling. Viral protein synthesis in vaccinia virus-infected cells is temporally regulated and essentially biphasic (for a review, see reference 22). Early viral proteins are detected immediately after infection, prior to viral DNA replication. During this prereplicative phase, host protein synthesis is also shut off. Late viral protein synthesis, on the other hand, is dependent upon the onset of viral DNA replication. During this postreplicative phase, late viral proteins are most abundant in infected-cell lysates, while early viral proteins rapidly decrease with time. Although viral DNA replication is essential for late viral protein synthesis, it is not sufficient. *ts* mutants have been described which are deficient in late protein synthesis, although they are competent for DNA replication at the nonpermissive temperature (7, 8). *ts*36 and *ts*50 were described as such (7, 8).

To monitor the pattern of viral protein synthesis, L-cell monolayers were infected with wt, *ts*36, or *ts*50 (MOI of 15) and pulsed with [^{35}S]methionine for 30 min at 1.5, 3, 4.5, 6, 7.5, and 9 h after infection. After cell lysis, the labeled polypeptides were separated by SDS-PAGE (Fig. 2). At the permissive temperature, both mutants demonstrated the

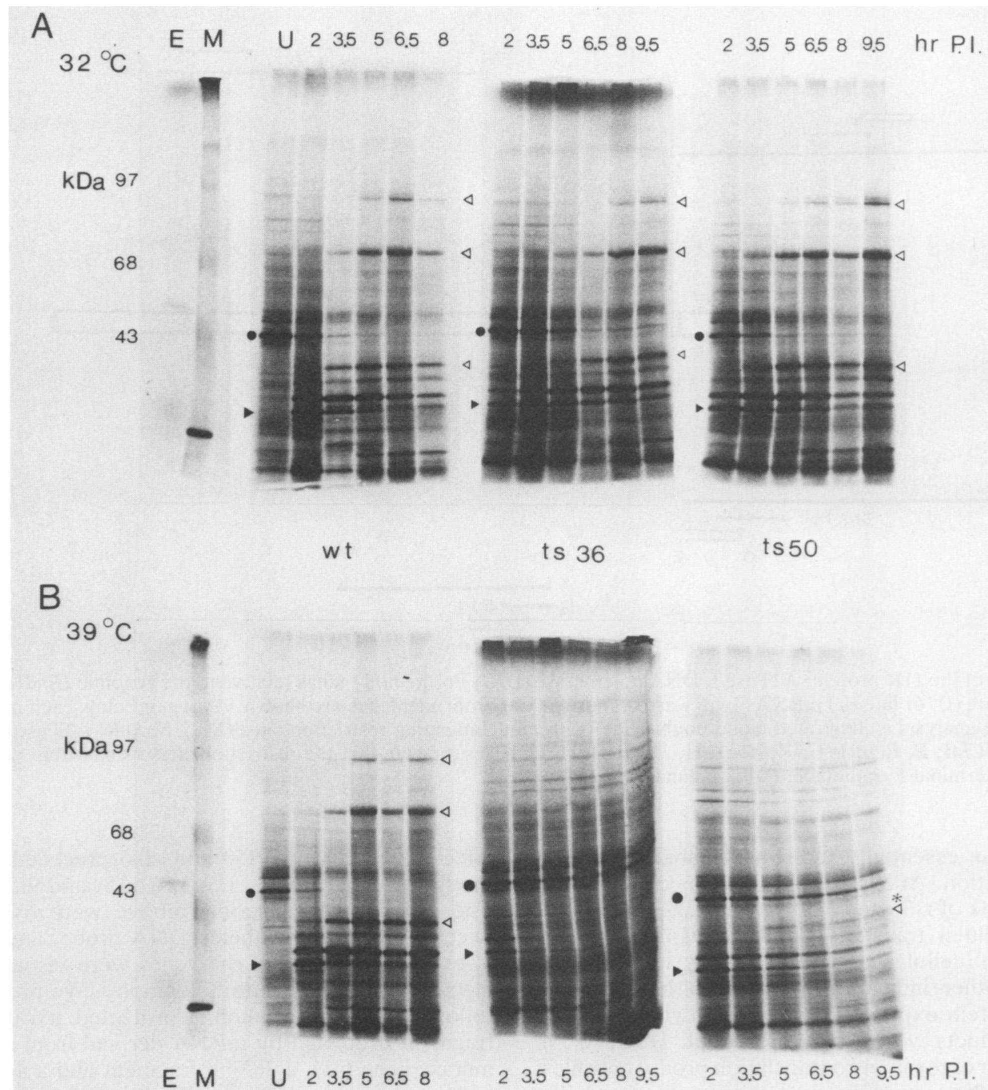

FIG. 3. In vitro translation of total RNA isolated from cells infected with wt, *ts*36, or *ts*60 virus (MOI of 15) and maintained at 32 and 39.5°C. Total RNA was isolated at the indicated times after infection. An aliquot from each sample was translated in a rabbit reticulocyte lysate system. The products of translation were separated by SDS-PAGE. Autoradiographs of the gels are shown. Lanes E, Translation products formed in the absence of added RNA; lanes U, translation products of RNA prepared from mock-infected cells; lanes M, migration of ^{14}C-labeled protein standards. Cellular actin (●), abundant early viral proteins (▶), and abundant late viral proteins (◁) are indicated. P.I., Postinfection.

same characteristic pattern of temporally regulated protein synthesis as wt vaccinia virus. However, the onset of late protein synthesis following infection with *ts*50 was somewhat delayed, as illustrated by the lysate labeled at hour 5 postinfection (Fig. 2A, compare lanes 5 for wt, *ts*36, and *ts*50). At the nonpermissive temperature, the viral protein synthesis profiles of both mutants were considerably altered with respect to that of the wt virus (Fig. 2B). In the mutant virus infections, viral proteins were set against a high background of host proteins, and the persistence of cellular actin was strongly indicative of this poor host protein synthesis shutoff (Fig. 2). Early viral protein synthesis was not down regulated in the postreplicative phase of infection (5 to 9.5 h postinfection), and there was a conspicuous absence of late proteins. It is also important to note that at this level of analysis, the protein profiles of *ts*36 and *ts*50 appeared identical.

Analysis of viral mRNAs by in vitro translation. Since *ts*36 and *ts*50 were shown to proceed normally from early viral protein synthesis to viral DNA replication, we wished to investigate whether the striking absence of late viral proteins was the result of a transcriptional or translational defect. To investigate the pool of mRNAs present in these cells, total RNA was isolated from mock-infected and infected cells at 2, 3.5, 5, 6.5, 8, and 9.5 h after infection. An aliquot of RNA from each time point was translated for 60 min at 37°C in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine. The translation products were separated by SDS-PAGE.

At both the permissive and nonpermissive temperatures, the protein expression profiles obtained by in vitro translation paralleled those obtained by metabolic labeling of infected cells (compare Fig. 2 and 3). At 32°C, early and late mRNAs were temporally regulated and the pattern of pro-

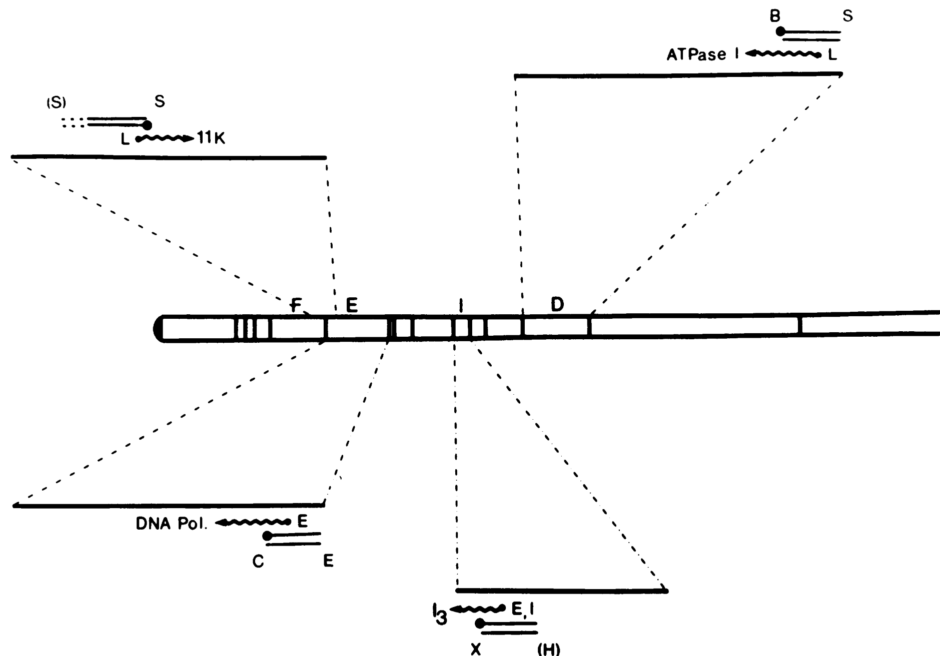

FIG. 4. Positions of the 11K protein, ATPase I, DNA polymerase (DNA Pol.), and I_3 genes relative to the genomic *Hin*dIII fragments. The early (E), intermediate (I), or late (L) mRNAs (wavy arrows) originating from each gene are shown. Above or below each mRNA, the probe used for S1 nuclease analysis is illustrated (solid double line) with its deliminating restriction sites (11K: S, *Sal*I; ATPase I: B, *Bam*HI; S, *Spe*I; DNA Pol.: C, *Cla*I; E, *Eco*RI; I_3: X, *Xba*I; H, *Hin*dIII). Parentheses indicate that the restriction sites were within vector sequences. The position of the terminal 5′ radiolabel (●) is indicated.

teins observed was essentially indistinguishable from that seen after wt infection. At 39.5°C, the virus-specific in vitro translation products of *ts*36 and *ts*50 were masked by a high background of cellular translation products (Fig. 3B), and the persistence of cellular actin throughout the time of infection was another indication of deficient host protein shutoff. Early protein expression was not down regulated, and late gene products were strikingly absent. However, a careful comparison of the in vitro translation product profiles of *ts*36 and *ts*50 at the nonpermissive temperature revealed a subtle difference between the two mutants. In the *ts*36 infection, we found some measure of regulation of early protein expression, as indicated by the time course of the early protein designated by a solid triangle (Fig. 3B, *ts*36), while in *ts*50, the appearance of a late viral protein (◁, with asterisk), albeit at very low levels compared with wt expression, indicated that the late protein synthesis defect in this mutant was not absolute. These data strongly suggest that the absence of late viral protein synthesis during 39.5°C infections with *ts*36 and *ts*50 (Fig. 2) reflected a lack of translatable late mRNAs. Thus, the barrier to the progression of viral infection is transcriptional and not translational.

Kinetic S1 nuclease analysis of four viral genes. In order to probe the transcriptional defect of the two mutants more finely and investigate further the nature of the difference between *ts*36 and *ts*50, we monitored the mRNAs from four different genes (Fig. 4) during a course of infection with wt or mutant virus at the nonpermissive temperature. With terminally labeled DNA probes, we used the S1 nuclease protection assay (44) to measure the steady-state levels of mRNAs derived from the genes encoding DNA polymerase, I_3 (a protein of the newly described intermediate class [43]), the 11K protein (a prototypic late gene preceded by a TAAAT motif [2, 14, 31]), and DNA-dependent ATPase I (the gene affected in *ts*36 and *ts*50 and also preceded by a TAAAT motif [5, 31]) at h 0, 2, 3.5, 5, 6.5, 8, and 9.5 postinfection. The same RNAs described above were hybridized to an excess of each radiolabeled DNA probe, and the protected 5′-terminal portions of the genes were visualized following electrophoresis and autoradiography. We predicted that the following fragments would be protected: a 600-base-pair (bp) fragment protected by mRNA derived from the DNA polymerase gene (41), a 365-bp fragment and a 400-bp fragment protected by two distinct mRNAs derived from the I_3 gene (43), a unique 290-bp fragment protected by mRNA derived from the 11K protein gene (45), and lastly a unique 545-bp fragment protected by an mRNA derived from the DNA-dependent ATPase gene (5, 30).

The fate of the above-mentioned mRNAs during 8 h of infection at 39.5°C with wt virus are shown in Fig. 5A through D. To our surprise, we detected two protected fragments for DNA polymerase with distinct temporal regulation (Fig. 5A). One appeared by 2 h postinfection and persisted, whereas the larger fragment was not seen before 3.5 h postinfection. A close examination of the published sequence of the DNA polymerase gene (11) revealed three potential TAAAT motifs in the region upstream of the initiating codon. One of them overlaps the predicted early transcription start site, and another is an additional 26 nucleotides upstream. The predicted fragments generated by protection of the probe by mRNAs initiating at these motifs would be 603 and 573 nucleotides long and are in excellent agreement with the fragments we observed. The time course for the shorter mRNA was consistent with that of an early transcript succeeded by a comigrating late transcript, while that for the longer mRNA was consistent with that of a late transcript (compare 5A and C). Both mRNAs remained at a steady level throughout the course of infection.

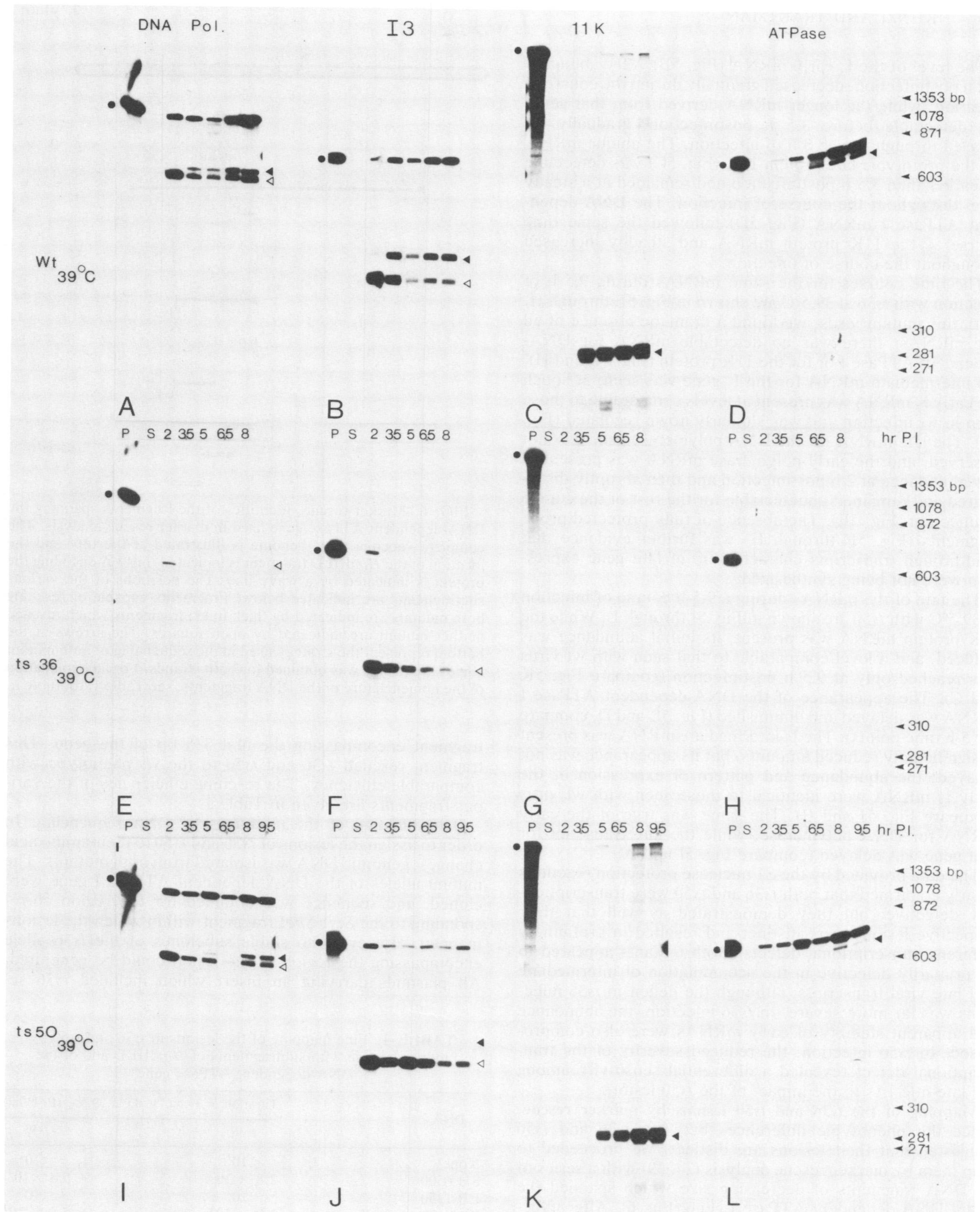

FIG. 5. S1 nuclease protection analysis. The products of S1 nuclease reactions (hybridization at 38°C) for the probes diagrammed in Fig. 4 are shown. (A, E, and I) DNA polymerase (DNA Pol.); (B, F, and J) I_3 probe; (C, G, and K) 11K probe; (D, H, and L) ATPase probe. Total RNA was isolated from L cells infected with wt, *ts*36, or *ts*50 virus (MOI of 15) at the nonpermissive temperature at the indicated hours postinfection (P.I.). For each reaction, aliquots (estimated to represent about 20 μg) of total RNA were hybridized under conditions of DNA excess. Lanes P, Migration of the probe (●) after incubation in the absence of RNA and without S1 digestion; lanes S, full digestion of the probe by S1 after incubation in the absence of RNA. Fragments protected by hybridization with early mRNAs (◁) and intermediate or late mRNAs (◀) are indicated.

The level of the I_3 early mRNA (Fig. 5B), very abundant at 2 h postinfection, decreased gradually during the course of infection, while the longer mRNA derived from that gene, not detectable before 3.5 h postinfection, gradually increased throughout the 8 h of infection. The unique mRNA for the prototypic late protein 11K (Fig. 5C) was detectable no earlier than 3.5 h postinfection and remained at a steady level throughout the course of infection. The DNA-dependent ATPase I mRNA (Fig. 5D) followed the same time course as the 11K protein mRNA and steadily increased throughout the course of infection.

The time courses for the same mRNAs during 9.5 h of infection with *ts*36 at 39.5°C are shown in Fig. 5E through H. With this mutant virus, we found a dramatic absence of all late mRNAs: there was no detectable mRNA for DNA-dependent ATPase I or for the 11K protein (Fig. 5G and H). No intermediate mRNA for the I_3 gene was seen, although the early I_3 mRNA was present at levels comparable to those seen in wt infection and was similarly down regulated (Fig. 5F). The late mRNA for the DNA polymerase gene was not observed, and the early polymerase mRNA was present at low abundance at 2 h postinfection and then abruptly disappeared and remained undetectable for the rest of the course of infection (Fig. 5E). The absence of fully protected probe fragments (Fig. 5E through H) was further evidence that readthrough transcripts characteristic of late gene expression were not being synthesized.

The fate of the mRNAs during a 9.5-h course of infection at 39.5°C with *ts*50 are shown in Fig. 5I through L. While the 11K protein mRNA was present, its initial abundance was reduced, and a level comparable to that seen with wt virus was reached only at 9.5 h postinfection (compare Fig. 5K and C). The appearance of the DNA-dependent ATPase I mRNA was delayed and diminished (Fig. 5L and D, compare the 5-h time points). The I_3 intermediate mRNA was present in significantly reduced amounts, but its appearance was not delayed; the abundance and pattern of expression of the early I_3 mRNA were identical to those seen with wt virus (compare Fig. 5J and B). The same was true of the early mRNA for DNA polymerase, while the late transcript for that gene was delayed (compare Fig. 5I and A).

The data provided by the S1 nuclease protection reactions clearly confirmed that both *ts*36 and *ts*50 were transcriptionally defective, but they demonstrated as well that their respective lesions were distinct and resulted in profoundly different transcriptional defects. Both mutants appeared to be primarily defective in the accumulation of intermediate and late viral transcripts, although the deficit in *ts*36 infections was far more severe. In *ts*36 infection, the abundance and apparent stability of early mRNAs were also compromised. In *ts*50 infection, the reduced severity of the transcriptional defect revealed a differential sensitivity among the late mRNAs to the impact of the *ts*50 lesion.

Mapping of the *ts36* and *ts*50 lesions by marker rescue. Since the phenotypic differences between *ts*36 and *ts*50 suggested that their lesions are distinct, we proceeded to map them by marker rescue analysis (24, 39) with a series of wt DNA fragments of decreasing size which spanned the entire DNA-dependent ATPase I gene (Fig. 6). After infection with the mutant virus and transfection with the appropriate wt DNA fragments, cultures were maintained at the nonpermissive temperature for 3 days. The ability of each fragment to rescue the mutant to the wt phenotype was monitored by assaying the viral yield at 39.5°C. The lesions of both *ts*36 and *ts*50 were localized within a 5′ *Bam*HI fragment of 700 bp and subsequently within a *Sty*I-*Spe*I fragment encompassing the first 335 bp of the gene. This fragment rescued *ts*36 and *ts*50 to the wt phenotype with comparable efficiency. The rescuing efficiencies of the various fragments are given in Table 1.

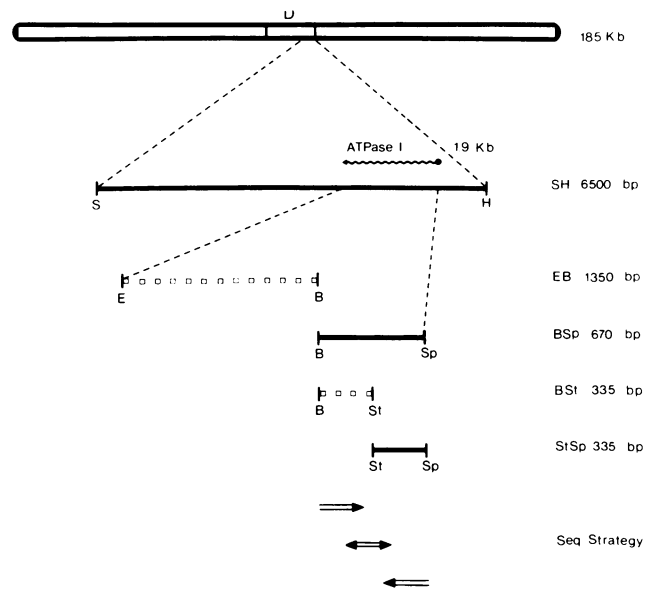

FIG. 6. Marker rescue. Identities of the fragments spanning the DNA-dependent ATPase gene used in marker rescue analysis. The complete vaccinia virus genome is illustrated at the top, and the location of the *Hin*dIII D fragment is indicated. mRNA encoding the protein is indicated by a wavy line. The positions of the various subfragments are indicated below. Fragments capable of rescuing both mutants are indicated by thick lines; fragments which rescued neither mutant are indicated by open squares. The arrows at the bottom represent the strategy used in the sequencing of both mutant alleles. Sequence was obtained for both strands. For an explanation of the nomenclature of the DNA fragments, see Table 1, footnote *a*.

Determination of the mutations by DNA sequencing. In order to assign the lesions of *ts*36 and *ts*50 to their amino acid changes, genomic DNA was isolated from both mutants. The mutant alleles of the DNA-dependent ATPase I gene were cloned, and sequence was obtained for the region corresponding to the *Sty*I-*Spe*I fragment within which the lesions mapped. However, no stable subclones of the *ts*36 allele encompassing the region of the lesion could be generated. All plasmids carrying an insert which included *ts*36 se-

TABLE 1. Fine mapping of the lesions in *ts*36 and *ts*50 by marker rescue with subfragments of *Hin*dIII-D and of the DNA-dependent ATPase gene[a]

DNA	Titration of 39.5°C yield at 39.5°C (PFU/ml)	
	*ts*36	*ts*50
None	$<10^1$	$<10^1$
SH 6500	2.8×10^5	9.4×10^4
EB 1350	$<10^1$	$<10^1$
BSp 670	1.9×10^3	5.0×10^3
BSt 335	$<10^1$	$<10^1$
StSp 335	1.0×10^3	1.2×10^3

[a] Marker rescue was performed as described in the text. The DNA fragments are named according to their delimiting restriction sites, from left to right, with respect to the vaccinia virus genome, and their sizes in base pairs. Their relative positions are shown in Fig. 6. S, *Sal*I; H, *Hin*dIII; E, *Eae*I; B, *Bam*HI; Sp, *Spe*I; St, *Sty*I.

```
                          20                                      40                                      60
            *              *              *              *              *              *
ATG AGT AAA TCA CAC GCG GCC TAT ATC GAT TAT GCA TTG CGC AGA ACT ACT AAT ATG CCT
Met Ser Lys Ser His Ala Ala Tyr Ile Asp Tyr Ala Leu Arg Arg Thr Thr Asn Met Pro
                          80                                     100                                     120
            *              *              *              *              *              *
GTT GAA ATG ATG GGG TCG GAC GTA GTA CGC CTC AAG GAT TAT CAA CAT TTT GTA GCA AGA
Val Glu Met Met Gly Ser Asp Val Val Arg Leu Lys Asp Tyr Gln His Phe Val Ala Arg
            Glu           140                                    160                                     180
            GAA            *              *              *              *              *
GTT TTC TTA GGA TTA GAC AGT ATG CAT TCT CTT TTA TTG TTC CAT GAA ACG GGT GTC GGT
Val Phe Leu Gly Leu Asp Ser Met His Ser Leu Leu Leu Phe His Glu Thr Gly Val Gly
                         200                                     220                                     240
            *              *              *              *              *              *
AAA ACA ATG ACT ACT GTA TAT ATT CTC AAA CAT CTT AAG GAT ATT TAT ACG AAT TGG GCT
Lys Thr Met Thr Thr Val Tyr Ile Leu Lys His Leu Lys Asp Ile Tyr Thr Asn Trp Ala
                         260                                     280                                     300
            *              *              *              *              *              *
ATT ATC TTA TTG GTG AAA AAG GCT TTG ATA GAA GAT CCT TGG ATG AAC ACT ATA CTC AGA
Ile Ile Leu Leu Val Lys Lys Ala Leu Ile Glu Asp Pro Trp Met Asn Thr Ile Leu Arg
                         320                                     340                                     360
            *              *              *              *              *              *
TAC GCT CCA GAG ATA ACG AAG GAT TGT ATT TTT ATT AAT TAC GAT GAT CAA AAT TTT AGA
Tyr Ala Pro Glu Ile Thr Lys Asp Cys Ile Phe Ile Asn Tyr Asp Asp Gln Asn Phe Arg
                         380                                     400                                     420
            *              *              *              *              *              *
AAT AAA TTT TTT ACT AAT ATC AAA ACT ATT CAT TCC AAG AGT AGA ATA TGC GTC ATT ATT
Asn Lys Phe Phe Thr Asn Ile Lys Thr Ile His Ser Lys Ser Arg Ile Cys Val Ile Ile
                         440                                     460                                     480
            *              *              *              *              *              *
GAT GAA TGT CAT AAC TTC ATT TCT AAA TCA ATA ATC AAA GAA GAT GGT AAG ATC CGT CAT
Asp Glu Cys His Asn Phe Ile Ser Lys Ser Ile Ile Lys Glu Asp Gly Lys Ile Arg His
                         500                                     520                                     540
            *              *              *              *              *              *
ACT CGT TCA GTA TAT AAT TTT TTA TCT AAG ACC ATC GCA TTA AAA AAC CAT AAG ATG ATT
Thr Arg Ser Val Tyr Asn Phe Leu Ser Lys Thr Ile Ala Leu Lys Asn His Lys Met Ile
                         560                                     580                                     600
            *              *              *              *              *              *
TGT TTA TCG GCT ACA CCT ATC GTC AAT AGT GTG CAA GAA TTC ACC ATG TTG GTT AAA TTA
Cys Leu Ser Ala Thr Pro Ile Val Asn Ser Val Gln Glu Phe Thr Met Leu Val Lys Leu

            *
CTA CGA CCA GGA TCC
Leu Arg Pro Gly Ser
```

FIG. 7. DNA sequence of the wt ATPase I gene (5, 30) and identification of the lesion in *ts*50. The first 615 nucleotides of the gene and the corresponding amino acids (1 to 205) are shown. The DNA region within which the lesion was localized by marker rescue (ATG to *Sty*I site [nucleotide 282]) is shown in boldface. The DNA sequences of both strands of the mutant allele were determined by the strategy indicated in Fig. 6 and as described in the text. The single nucleotide and amino acid changes observed are indicated above the wt sequence (box). The region of homology which is conserved in a variety of ATP-binding proteins (5) is underlined.

quences corresponding to the *Sty*I-*Spe*I fragment grew poorly or not at all. Those plasmids which could be isolated were found to be wt in sequence after amplification in *E. coli* and were able to restore the mutant to the wt phenotype. Analysis of the *ts*50 allele, on the other hand, presented no such difficulties, and sequence was obtained for a 700-bp fragment which encompassed the *Sty*I-*Spe*I fragment (Fig. 6). Within the *ts*50 coding sequence, we found a single base-pair mutation of a G (base 71) to an A, resulting in an amino acid change of a glycine, a polar uncharged residue, to a glutamic acid, an acidic residue (Fig. 7, box). The mutation was found to be within 8 amino acid residues of a region of homology (underlined bases) which is conserved in a variety of ATP-binding proteins (5). The altered glycine residue, interestingly, is conserved in RecA.

DISCUSSION

*ts*36 and *ts*50 are conditional lethal mutants of vaccinia virus which bear lesions in a gene encoding a DNA-dependent ATPase. Biochemical studies of the ATPase have shown that the 68-kDa protein will hydrolyze ATP or dATP in the presence of a nucleic acid cofactor (26 and data not shown). The encapsidation of the ATPase in the viral core suggested a role for the enzyme in early gene expression by analogy with other virion enzymes such as the RNA polymerase, poly(A) polymerase, capping enzyme, and topoisomerase. Our attempts to detect more specific biochemical properties, such as helicase activity, have been unsuccessful. Broyles and Moss (4; B. Moss, personal communication), studying a purified transcriptional complex competent to direct accurate transcription on a linear DNA template, could not confirm any consistent association with, or requirement for, the ATPase.

To delineate the physiological role of the enzyme in the life cycle of the virus, we undertook a more detailed analysis of the phenotypes of *ts*36 and *ts*50. The initial characterization of these mutants (7, 8) indicated that early gene expression was unimpaired; DNA synthesis, as measured by the

incorporation of [^{3}H]thymidine into acid-precipitable material, also appeared unaffected. Our quantitative analysis of the accumulation of viral DNA sequences following infection confirmed that equivalent amounts of genomic DNA were synthesized at 32 and 39.5°C. However, cells infected with *ts*36 exhibited a delay of approximately 3 h in the onset of DNA synthesis (relative to a wt virus infection) at both permissive and nonpermissive temperatures.

Analysis of the patterns of protein synthesis in infected cells was also undertaken. At the permissive temperature, the pattern in *ts*36-infected cells was indistinguishable from that seen with wt virus, whereas in *ts*50-infected cells the appearance of late viral proteins was somewhat delayed. Thus, both *ts*36 and *ts*50 appear to show some subtle delays in DNA synthesis or late gene expression even at 32°C. The data obtained at 39.5°C confirmed the classification of these mutants as possessing a defective late phenotype. The synthesis of early viral proteins continued throughout infection, and no late viral proteins were detected. Moreover, the synthesis of host proteins persisted for up to 9.5 h postinfection. In vitro translation of RNA isolated at various times after infection revealed that the deficit in late protein synthesis reflected the nearly complete absence of translatable late viral mRNAs. It is worth noting, however, that very low levels of a late viral protein could be detected after in vitro translation of RNA harvested from *ts*50-infected cells at 39.5°C. Thus, a net transcriptional defect, rather than a lesion in the translational machinery, appears to be involved.

A finer analysis of selected viral mRNAs was accomplished using S1 nuclease mapping. During wt virus infection, the DNA polymerase gene appeared to be transcribed both early and late; one late transcript comigrated with the early transcript, and a second was distinct and initiated further upstream. Late transcripts appeared by 3.5 (but not 2) h postinfection. Distinct early and late transcripts originating from the I_3 gene were detected; recent work by Vos and Stunnenberg (43) suggests that this late transcript may in fact represent a newly defined class of intermediate genes derepressed in *cis* upon DNA synthesis but expressed by early *trans*-acting machinery. The 11K and ATPase transcripts displayed the typical late pattern of expression.

*ts*36-infected cells showed a drastic deficit in late transcript accumulation during infection at 39.5°C. No evidence was found of the late DNA polymerase, 11K, or ATPase transcripts. Interestingly, the purported intermediate transcript from the I_3 gene was also absent. The phenotype of *ts*50 appeared far less severe in this assay. The appearance of the late transcripts from the DNA polymerase, 11K, and ATPase genes was delayed by approximately 1.5 h; however, significant amounts of DNA polymerase and 11K transcripts did accumulate. The I_3 intermediate transcript showed normal kinetics but was barely detectable. This discrepancy between *ts*36 and *ts*50 was striking in the light of their nearly indistinguishable phenotypes as measured by the criteria of plaque-forming ability, protein synthesis, and in vitro translation of isolated RNA. Whereas *ts*36 revealed a complete deficit in the accumulation of intermediate and late transcripts, *ts*50 showed a diminution in intermediate transcripts and a delay in late transcripts.

The combined data suggest that the ATPase may play a role in intermediate and/or late gene expression. If intermediate gene products play an obligate role in the switch to late gene expression, then their absence would arrest the progression of the viral life cycle. Similarly, their diminution might delay the expression of some late transcripts and prevent the expression of other, crucial genes. A deficit in intermediate gene expression might reflect a defect in the *trans*-acting transcriptional machinery or in the process by which DNA replication derepresses intermediate genes in a *cis*-acting manner. Although the same *trans*-acting machinery may be involved in the expression of early and intermediate genes, the details of vaccinia virus biology may explain the differential impact of the *ts* mutations.

The enzyme is brought into infected cells in the viral core and is then expressed de novo late in infection. Even during infections performed at the nonpermissive temperature, entering virions contain ATPase which was obligatorily synthesized and packaged during a previous round of infection at the permissive temperature. A recent study of viral mutants with temperature-sensitive lesions in RNA polymerase subunits (41) indicates that synthesis and encapsidation at the permissive temperature mask the temperature-sensitive phenotype during the early phase of infection. If the DNA-dependent ATPase plays an essential role in early gene expression, its prior synthesis and packaging at 32°C might mask its temperature sensitivity. The defect might be revealed only following the uncoating that precedes DNA replication (and intermediate gene expression) and/or following de novo synthesis of the enzyme at 39.5°C during the late phase of infection.

Our analysis of these mutants included the fine mapping of their lesions. Marker rescue experiments localized both mutations within the same 335-bp fragment at the 5′ end of the gene. Genomic DNA was isolated from both viruses to enable molecular cloning and sequencing of the mutant alleles. Although genomic DNA could be reproducibly isolated from *ts*36-infected cells, no stable clones which retained the mutation could be generated. Difficulty was encountered in growing plasmids containing the *ts*36 ATPase allele in *E. coli*. Transformants grew poorly if at all, and plasmids which were eventually isolated were wt in sequence and had regained the ability to restore *ts*36 to the wt phenotype in marker rescue studies. Although we cannot fully explain our inability to stably propagate the *ts*36 allele, a reasonable explanation is that the altered sequence is either toxic or unstable in *E. coli*. Because the viral sequences were not engineered behind a bacterial promoter and because recognition of late vaccinia virus promoters in *E. coli* has not been documented, it is likely that the incompatibility is at the level of the DNA sequence and not expressed protein. Either only rare transformants containing revertant alleles of the *ts*36 gene survive or bacterial repair processes alter the sequence in a manner which fortuitously restores the wt sequence. No such difficulties were encountered with *ts*50. DNA sequence was obtained for approximately 700 bp which included the region known to contain the lesion. A single nucleotide change was found, which resulted in the replacement of a glycine residue with a glutamic acid residue. This substitution mapped within 8 residues of the consensus ATP-binding site located in this 5′-terminal region of the ATPase.

Our results indicate that the ATPase is likely to play a role in regulating or facilitating viral transcription. Comparison of the ATPase sequence with the available protein sequence data bases did not provide any insight into the biochemical function of the enzyme. However, other DNA-dependent ATPases with roles in transcription have been shown to be RNA-DNA helicases (3, 35) or transcription factors (1, 6). Overexpression and comparison of the wt- and *ts*50-encoded enzymes should allow a refined understanding of the interactions of the enzyme with the nucleic acid cofactor. In conjunction with further in vivo studies, these analyses

should clarify the essential role of the ATPase in the progression of the vaccinia virus replicative cycle.

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