JOURNAL OF VIROLOGY, June 1987, p. 1842–1850
0022-538X/87/061842-09$02.00/0

Vol. 61, No. 6

Phenotypic Characterization of Temperature-Sensitive Mutants of Vaccinia Virus with Mutations in a 135,000-M_r Subunit of the Virion-Associated DNA-Dependent RNA Polymerase

MARCIA J. ENSINGER

The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

Received 9 October 1986/Accepted 22 February 1987

The phenotypic defects of three temperature-sensitive (*ts*) mutants of vaccinia virus, the *ts* mutations of which were mapped to the gene for one of the high-molecular-weight subunits of the virion-associated DNA-dependent RNA polymerase, were characterized. Because the virion RNA polymerase is required for the initiation of the viral replication cycle, it has been predicted that this type of mutant is defective in viral DNA replication and the synthesis of early viral proteins at the nonpermissive temperature. However, all three mutants synthesized both DNA and early proteins, and two of the three synthesized late proteins as well. RNA synthesis in vitro by permeabilized mutant virions was not more *ts* than that by the wild type. Furthermore, only one of three RNA polymerase activities that was partially purified from virions assembled at the permissive temperature displayed altered biochemical properties in vitro that could be correlated with its *ts* mutation: the *ts*13 activity had reduced specific activity, increased temperature sensitivity, and increased thermolability under a variety of preincubation conditions. Although the partially purified polymerase activity of a second mutant, *ts*72, was also more thermolabile than the wild-type activity, the thermolability was shown to be the result of a second mutation within the RNA polymerase gene. These results suggest that the defects in these mutants affect the assembly of newly synthesized polymerase subunits into active enzyme or the incorporation of RNA polymerase into maturing virions; once synthesized at the permissive temperature, the mutant polymerases are able to function in the initiation of subsequent rounds of infection at the nonpermissive temperature.

The vaccinia virus virion contains a number of enzyme activities that are involved in mRNA biosynthesis, including a DNA-dependent RNA polymerase and capping, methylating, and polyadenylating enzymes (for reviews, see references 11 and 29). Viral cores prepared in vitro by treatment of purified virions with a nonionic detergent and a reducing agent synthesize RNA that is similar to in vivo mRNA. It is capped at the 5′ end, is poly(A)$^+$ at the 3′ end, is similar in size, and can be translated in vitro to yield authentic viral proteins. In vivo, early viral mRNA is made by the enzymes within partially uncoated virions, and thus their action is required for the initiation of the viral replication cycle. The isolation of conditionally lethal mutants with defects in the virion-associated enzymes would be instrumental in understanding the molecular mechanisms of mRNA biogenesis. Because of the role that these enzymes play in the initiation of infection, it has been predicted (37) that this type of mutant would not induce the synthesis of early viral proteins or replicate viral DNA under nonpermissive conditions. Less than 10% of the temperature-sensitive (*ts*) mutants in any one collection are DNA-minus, however; and no *ts* mutants have been identified thus far that fail to induce the synthesis of early viral proteins at the nonpermissive temperature (7–10, 12, 13, 25). Thus, either the techniques that were employed to isolate *ts* mutants selected against virion-associated enzyme mutants or the enzyme mutants that were generated did not have the predicted phenotype.

The vaccinia virus RNA polymerase is a complex enzyme composed of seven to eight subunits, two of which have molecular weights of approximately 135,000 (135K subunits) (2, 32, 36). In two laboratories the gene for one of the 135K subunits has been mapped to the region of the *Hin*dIII-J and -H restriction endonuclease site on the vaccinia virus DNA (5, 28). A single early mRNA with a translational product that was previously estimated to be a 110K subunit has been mapped precisely within this region (1, 26). I report here the phenotypic characterization of three mutants, *ts*8, *ts*13, and *ts*72, in one complementation group with *ts* lesions that have been mapped to the region of the *Hin*dIII-J and -H junction (16). The mutations in *ts*8 and *ts*13 have been previously shown to lie within the gene for the RNA polymerase 135K subunit (16), and here I show that the mutation in *ts*72 also lies within this gene. Contrary to the prediction that RNA polymerase mutants are defective in the early stages of the replication cycle, these mutants did synthesize early viral proteins and DNA at the nonpermissive temperature, and two of the three synthesized late proteins. Furthermore, biochemical studies with the RNA polymerase that was partially purified from virions produced at the permissive temperature revealed that only one of the mutant RNA polymerase activities is *ts* in vitro. Thus, it is likely that the primary effect of the *ts* defects of these mutants is on the assembly of newly synthesized RNA polymerase subunits into active enzyme or the assembly of the RNA polymerase into maturing virions.

MATERIALS AND METHODS

Cells, virus, and virus purification. The isolation of *ts* mutants of vaccinia virus strain WR, preparation of viral stocks, and virus titration by plaque assay employing BSC40 cells have been described elsewhere (13). Virions were purified from suspension cultures of HeLa cells infected at 33°C by two sequential sucrose gradient sedimentations (22).

Measurement of viral DNA replication by dot blot hybridization. Monolayers of BSC40 cells in tissue culture dishes (diameter, 35 mm; 10^6 cells per dish) were infected with 10 PFU of mutant or wild-type virus per cell and incubated at 33 or 39.5°C. At various times after infection, the cells were

scraped from the dishes, centrifuged, washed once with phosphate-buffered saline, and suspended in 0.3 ml of loading buffer (10× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] containing 1 M ammonium acetate). The samples were frozen and thawed three times in a dry ice-ethanol bath and diluted with 4 volumes of loading buffer. A 0.1-ml portion was loaded onto a nitrocellulose filter, which had been presoaked in loading buffer, by using a microsample filtation manifold (Schleicher & Schuell, Inc., Keene, N.H.). The DNA was denatured by soaking the filter in 0.5 N NaOH and 1.5 M NaCl. After two successive washes in 1 M Tris hydrochloride (pH 7.5)–1.5 M NaCl, the filter was dried, baked at 80°C for 2 h in a vacuum oven, and then hybridized to an excess of the cloned vaccinia virus *Hin*dIII D fragment (3) that had been labeled with [^{32}P]CTP by nick translation (35). The hybridization conditions described by Thomas (38) were used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins. Monolayers of BSC40 cells in Eagle minimal essential medium containing 5% fetal calf serum and one-third the normal concentration of methionine were infected with 10 PFU per cell and incubated at 33 or 39.5°C. At various times after infection, the monolayers were washed with methionine-free medium and then incubated for 30 min in 0.5 ml of prewarmed methionine-free medium containing 50 μCi of [^{35}S]methionine (1,000 Ci/mmol) per ml. At the end of the labeling period, the cells were washed twice with ice-cold phosphate-buffered saline and then lysed by the addition of 0.1 ml of RIPA buffer (50 mM Tris hydrochloride [pH 7.2], 0.15 M NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], and 1% sodium deoxycholate [17]). The lysate was scraped into a centrifuge tube, frozen and thawed five times in a dry ice-ethanol bath, and centrifuged for 15 min at 10,000 rpm in a Sorvall rotor. The supernatant was carefully removed, and a portion of each sample representing an equivalent number of cells was electrophoresed on 10% SDS-polyacrylamide gels by using the discontinuous buffer system described by Laemmli (24).

Assay of permeabilized virions for RNA polymerase, mRNA methyltransferase, and poly(A) polymerase activities. Purified mutant or wild-type virions were incubated in a standard reaction mixture (0.1 ml) containing 50 mM Tris hydrochloride (pH 8.4), 5 mM dithiothreitol, 0.05% Nonidet P-40, and 0.4 A_{260} units of virus (39). The following additional components were added to specific reaction mixtures: (i) DNA-dependent RNA polymerase–2 mM ATP, 1 mM each of CTP and GTP, 0.5 mM [^{3}H]UTP (20 mCi/mmol), and 4.5 mM $MgCl_2$ (39); (ii) mRNA methyltransferase–2 mM ATP; 1 mM each of CTP, GTP, and UTP; 1 μM *S*-adenosyl[*methyl*-^{3}H]methionine (10 Ci/mmol); and 5 mM $MgCl_2$ (39); (iii) poly(A) polymerase–0.5 mM [^{3}H]ATP (10 mCi/mmol), 0.5 mM $MnCl_2$, and 0.1 mM poly(dA)-poly(dT) as primer (30). Reactions were incubated at 33 or 39.5°C for 30 min, and 75 μl was spotted onto DEAE-cellulose filter paper disks to measure the incorporation of radioactivity into macromolecular material (4).

Partial purification of RNA polymerase. RNA polymerase activity was partially purified from approximately 100 A_{260} units of purified mutant or wild-type virions following the initial steps of the procedure described by Baroudy and Moss (2). Briefly, virions were disrupted with sodium deoxycholate and dithiothreitol, centrifuged to remove insoluble proteins, and passed through a DEAE-cellulose column in buffer with a high salt concentration to remove DNA. The flowthrough from the DEAE-cellulose column was diluted to contain 50 mM NaCl in 50 mM Tris hydrochloride (pH 8.5)–1 mM dithiothreitol–0.1% Triton X-100–10% glycerol and applied to a DEAE-Sepharose column in the same buffer. The column was washed with additional buffer containing 50 mM NaCl followed by 0.1 M KCl, and then the RNA polymerase activity was step-eluted with buffer containing 0.2 M KCl. Fractions containing the polymerase activity were pooled and stored at −70°C. These partially purified preparations contained approximately 3 to 4% of the initial virion protein, with 100% or greater recovery of activity.

Assay of solubilized RNA polymerase, mRNA methyltransferase, and poly(A) polymerase activities. (i) For the RNA polymerase assay, reaction mixtures (0.1 ml) contained 50 mM Tris hydrochloride (pH 7.9); 2 mM dithiothreitol; 1 mM ATP, CTP, and GTP; 0.04 mM [^{3}H]UTP (0.25 Ci/mmol); 4 mM $MnCl_2$, 90 mM KCl; and 50 μg of denatured calf thymus DNA per ml (2). (ii) For the mRNA methyltransferase assay, reaction mixtures contained 50 mM Tris hydrochloride (pH 7.5), 1 mM dithiothreitol, 1 mM GTP, 1 μM *S*-adenosyl [*methyl*-^{3}H]methionine (10 Ci/mmol), 1 mM $MgCl_2$, and 50 μg of unmethylated vaccinia virus in vitro-synthesized mRNA per ml as substrate (14). (iii) For the poly(A) polymerase assay, reaction mixtures contained 50 mM Tris hydrochloride (pH 8.5), 1 mM dithiothreitol, 1 mM [^{3}H]ATP (10 mCi/mmol), 1 mM $MnCl_2$, and 0.1 mM poly(dA-dT) as primer (30). All reactions were incubated at the indicated temperatures for 30 min, and 75 μl was spotted onto DEAE-cellulose filter paper disks to determine the incorporation into macromolecular material (4).

Thermal inactivation of RNA polymerase activity. Partially purified RNA polymerase activities were incubated at the indicated temperatures in the standard reaction mixture containing 10% glycerol and from which DNA, all four ribonucleoside triphosphates, or both DNA and ribonucleoside triphosphates were omitted. Samples (50 μl) were withdrawn and mixed at 4°C with an equal volume of the standard reaction mixture containing twice the normal concentration of the component that was omitted from the preincubation mixture. The samples were kept on ice for 30 min and then incubated at 33°C for 30 min to determine the amount of residual polymerase activity.

Marker rescue. Marker rescue employing cloned restriction endonuclease fragments of wild-type vaccinia virus DNA was performed by the two-step procedure described previously (15). Briefly, monolayers of BSC40 cells were infected with *ts*72 and then transfected with calcium phosphate-precipitated recombinant DNA. The cultures were incubated at 39.5°C (selective conditions) or at 33°C (nonselective conditions), and the final yield of virus was titrated at 33 and 39.5°C to determine the proportion of wild-type recombinants. The *Hin*dIII-H–*Aha*III and *Hin*dIII-H–*Ava*II DNA fragments used in marker rescue were obtained from S. Broyles and B. Moss (National Institutes of Health, Bethesda, Md.). They were purified by agarose gel electrophoresis after restriction endonuclease cleavage of cloned *Hin*dIII-H.

RESULTS

Viral DNA synthesis in mutant-infected cells. Vaccinia virus DNA replication takes place in discrete areas, termed factories, in the cytoplasm of infected cells (6). I have shown previously (13), by using the DNA binding dye Hoechst 33258, that cytoplasmic DNA factories appear normally in cells that are infected by all three mutants in this complementation group. However, because of the prediction that mutants with lesions in one of the virion-associated enzyme

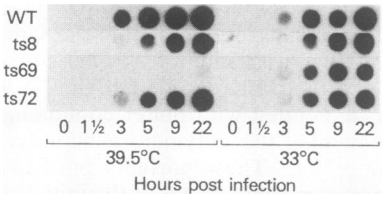

FIG. 1. Dot-blot analysis of virus-specific DNA accumulation in mutant- and wild-type-infected cells. BSC40 cells were infected with *ts* mutants or the wild type (WT) and incubated at 33 or 39.5°C. At the indicated times the cells were scraped from the dishes and disrupted by freezing and thawing. A portion of each sample was loaded onto a nitrocellulose filter, treated with NaOH to denature the DNA, and hybridized to a ^{32}P-labeled recombinant vaccinia virus *Hin*dIII-D DNA fragment, as described in the text. An autoradiogram is shown. *ts*69 is a DNA-minus mutant (13).

activities would be deficient in the synthesis of viral DNA, I reexamined this question more quantitatively by following the accumulation of viral-specific DNA sequences in mutant-infected cells. Total cell lysates prepared at various times after infection were hybridized to an excess of nick-translated vaccinia virus-specific probe by using a dot blot filter hybridization technique (see above). Control experiments indicated that the probe was in excess and that the hybridization was due to the DNA and not to the RNA that was present in the cell lysates (data not shown). The kinetics of accumulation of virus-specific DNA sequences in *ts*8-, *ts*72-, and wild-type-infected cells are shown in Fig. 1. The results obtained with a DNA-minus mutant (*ts*69) are shown for comparison. Although the previous observation that *ts*8 and *ts*72 are able to replicate their DNA at the nonpermissive temperature was confirmed, there was a delay in the appearance of viral-specific DNA sequences as well as a reduction in the final level that was achieved relative to that in wild-type-infected cells. However, because these differences were also seen to a lesser extent in cells that were infected by these mutants at 33°C, it is unlikely that this partial deficit in DNA replication is a major factor in the *ts* replication of these mutants. In earlier experiments in which pulses of [^{3}H]thymidine were employed, DNA replication in cells that were infected with *ts*13 was found to be similar to that seen in *ts*8- and *ts*72-infected cells (data not shown).

Protein synthesis in mutant-infected cells. Viral protein synthesis in vaccinia virus-infected cells takes place in two phases: an early phase that occurs before the onset of viral DNA replication and a late or postreplicative phase. If viral DNA replication is prevented either by using drugs that inhibit DNA synthesis or by infecting cells with mutants that are unable to replicate viral DNA under nonpermissive conditions, only early polypeptides are made. Because the three mutants in this complementation group were able to replicate viral DNA at the nonpermissive temperature, I next asked if the switch from the early to late pattern of polypeptide synthesis occurred normally in mutant-infected cells at 39.5°C. Monolayers of BSC40 cells were pulsed with [^{35}S]methionine at various times after infection with mutant or wild-type virus, and the labeled polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis, as described above. Each mutant gave a distinct pattern of polypeptide synthesis at 39.5°C (Fig. 2). In *ts*8-infected cells, the pattern of polypeptide synthesis was almost identical to that in wild-type-infected cells, although a slight delay in the appearance of the late polypeptides was noted when the samples labeled at 3 h postinfection were compared. In cells infected with *ts*13 at 39.5°C, the delay in the synthesis of late polypeptides was more dramatic; early polypeptides appeared as usual at 1 h after infection, but late polypeptides were not synthesized until 7 h postinfection. In addition, there was prolonged synthesis of host polypeptides. However, both these differences were noted in cells that were infected with *ts*13 at 33°C as well. The greatest difference from the wild-type pattern of polypeptide synthesis was seen in cells that were infected with *ts*72 at 39.5°C. The synthesis of late polypeptides was greatly reduced, and host polypeptide synthesis continued unabated for 24 h. At 33°C the pattern of polypeptide synthesis was normal. Thus, *ts*72 (and, to a lesser extent, *ts*13) resembles the defective late class of mutant described by Condit and Motyczka (8) and by Lake and Cooper (25). Although the defective late mutants described by Condit and Motyczka (8) did shut off host protein synthesis, and *ts*72 did not, this difference may be a reflection of the higher multiplicity of infection (20 PFU per cell) that was used in their experiments compared with the 10 PFU per cell used in my experiments.

RNA synthesis by permeabilized virions in vitro. The fact that all three of the RNA polymerase mutants synthesized DNA at the nonpermissive temperature suggests that once virions are assembled in cells that are infected at the permissive temperature, the RNA polymerase activity is stabilized by association with other polymerase subunits, other virion proteins, or DNA in the virus core and thus is able to function in the initiation of subsequent rounds of infection at 39.5°C. To test this hypothesis directly, mutant and wild-type virions purified from cells infected at 33°C were assayed for their ability to synthesize RNA in vitro at 33 and 39.5°C. All three mutants incorporated [^{3}H]UMP into macromolecular material equally well at 39.5°C relative to that at 33°C (Table 1). However, *ts*13 virions contained only one-fourth of the RNA polymerase activity that was found in the wild type. To determine if the reduced incorporation of radioactivity into RNA by *ts*13 virions was a direct effect of the mutation in the RNA polymerase or a more general virion defect, I assayed permeabilized virions for two other enzyme activities that are involved in the production of mature mRNA: mRNA methyltransferase in an assay that is dependent on transcription for the generation of the substrate (39) and poly(A) polymerase in an assay that is independent of transcription with poly(dA-dT) as primer (30). Both of these activities were present in *ts*8 and *ts*72 virions in quantities that were similar to those present in wild-type virions (Table 1). In *ts*13 virions, however, the methyltransferase activity was reduced, while the poly(A) polymerase activity was closer to the level observed in the wild-type virions. SDS-polyacrylamide gel electrophoresis of virion polypeptides revealed that *ts*13 virions contain an equivalent amount of the high-molecular-weight subunits of the RNA polymerase relative to that of the wild type (data not shown). Because virions purified from *ts*13-infected cells always had a three- to fivefold higher particle-to-PFU ratio than the wild type, these results suggest that the noninfectious particles contain reduced or inactive RNA polymerase activity.

Biochemical characterization of the RNA polymerase activity solubilized from mutant and wild-type virions. To investigate the biochemical properties of the RNA polymerase of these mutants, the enzyme activities were partially purified from disrupted virions following the initial steps of the protocol described by Baroudy and Moss (2) (see above). The specific activity of the partially purified RNA polymerase of *ts*13 was only 16% of the wild type activity, whereas

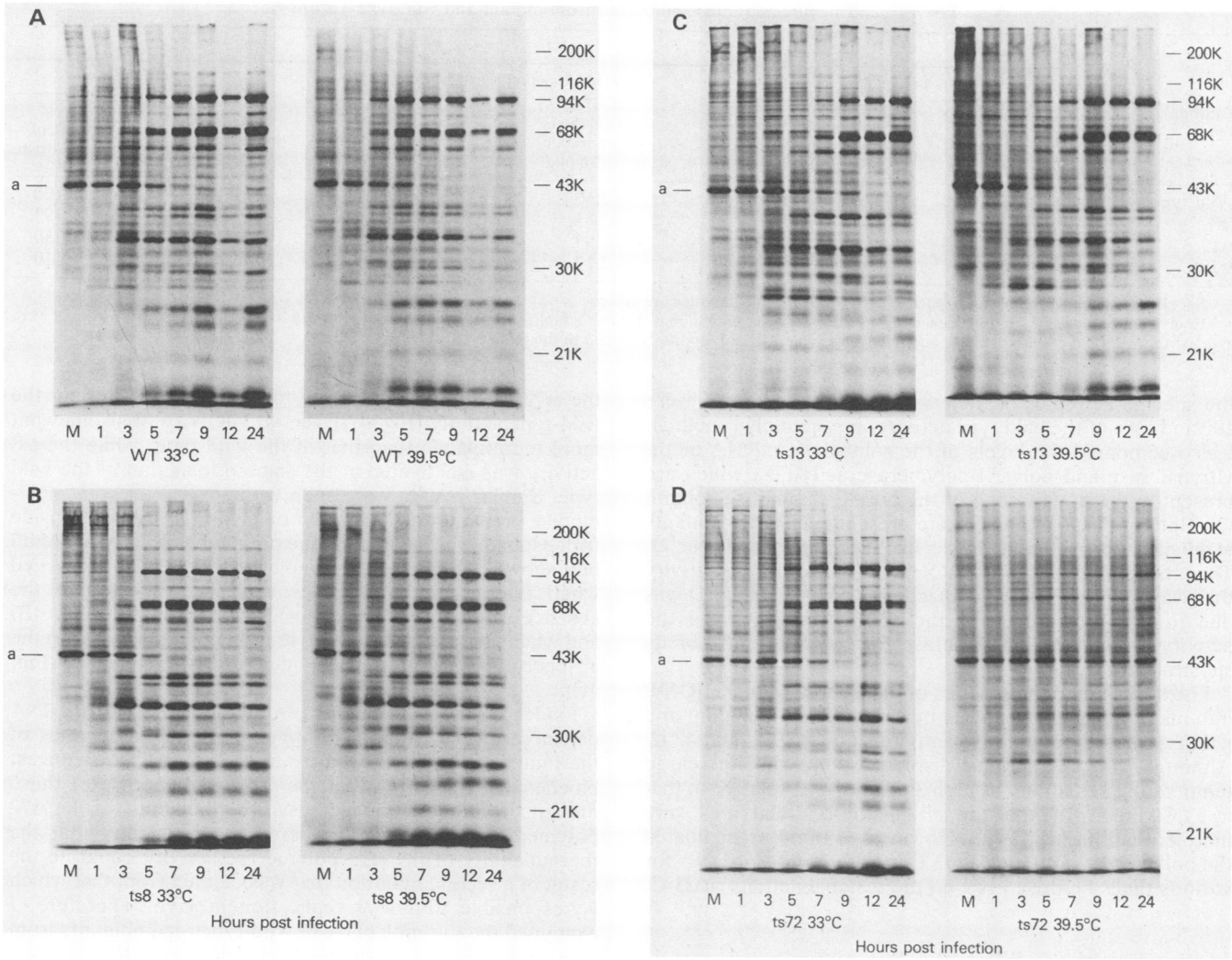

FIG. 2. Protein synthesis in *ts* mutant- and wild-type-infected cells. Cells were infected with *ts* mutants or wild-type (WT) vaccinia virus and pulsed for 30 min with [^{35}S]methionine at the indicated times. Labeled polypeptides were electrophoresed on 10% SDS-polyacrylamide gels (20 cm) as described in the text. Autoradiograms are shown. M denotes a lysate from mock-infected cells. The migration of molecular weight markers is indicated on the right, and the migration of cellular actin is indicated by the a on the left.

TABLE 1. Enzyme activities in permeabilized mutant and wild-type virions

Strain	Assay temp (°C)	Activity					
		RNA polymerase		mRNA methyltransferase		Poly(A) polymerase	
		[^{3}H]UMP incorporation (pmol/30 min)	% of wild type	[^{3}H]methyl incorporation (pmol/30 min)	% of wild type	[^{3}H]AMP incorporation (pmol/30 min)	% of wild type
Wild type	39.5	534	100	2.66	100	714	100
	33	513	100	3.42	100	681	100
*ts*8	39.5	519	97	2.40	90	693	97
	33	498	97	3.19	93	626	92
*ts*13	39.5	130	24	1.23	46	495	69
	33	142	28	1.23	36	450	66
*ts*72	39.5	434	81	2.22	83	541	76
	33	440	86	2.81	82	521	77

TABLE 2. Enzyme activities solubilized from mutant and wild-type virions

Strain	Activity					
	RNA polymerase[a]		mRNA methyltransferase[b]		poly(A) polymerase[b]	
	[^{3}H]UMP incorporation (pmol/30 min per mg of protein)	% of wild type	[^{3}H]methyl incorporation (pmol/30 min)	% of wild type	[^{3}H]AMP incorporation (pmol/30 min)	% of wild type
Wild type	1.74×10^5	100	2.66	100	269	100
*ts*8	1.78×10^5	100	2.83	110	249	93
*ts*13	2.87×10^4	16	1.99	75	361	130
*ts*72	8.89×10^4	51	2.19	82	294	110

[a] Specific activities of the partially purified RNA polymerase activities are given.
[b] Equivalent portions of the flowthrough of the DEAE-Sepharose column chromatography step during purification of the RNA polymerase from virions were assayed for mRNA methyltransferase and poly(A) polymerase activity, as described in the text.

the specific activities of the *ts*8 and *ts*72 polymerases were 100 and 51% of the wild type activity, respectively (Table 2). I also compared the levels of the solubilized mRNA methyltransferase and poly(A) polymerase activities, which are present in the flowthrough of the DEAE-Sepharose column (2). Both of these activities were present in all three mutants at levels approximating those in the wild type (Table 2). Thus, the reduction of the mRNA methyltransferase activity that was seen with permeabilized *ts*13 virions (Table 1) was due to the dependence of the methyltransferase on the activity of the RNA polymerase for the generation of its substrate.

I next examined the kinetics of incorporation of [^{3}H]UMP into macromolecular material by the solubilized mutant and wild-type RNA polymerase activities at 33, 39.5, and 42°C. Although the *ts*13 activity displayed somewhat increased temperature sensitivity at both 39.5 and 42°C relative to that at 33°C (Fig. 3), neither the *ts*8 nor the *ts*72 activities were more *ts* than the wild type. However, if I first preincubated the polymerase activities at 43°C in the absence of DNA for various times before I assayed the residual activity at 33°C,

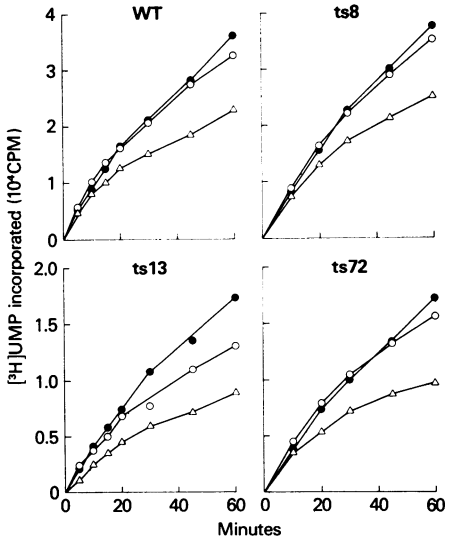

FIG. 3. Time course of incorporation of [^{3}H]UMP by mutant and wild-type (WT) RNA polymerase activities at 33, 39.5, and 42°C. Standard reaction mixtures containing either 3.5 μg (wild type, *ts*8, and *ts*72) or 7.1 μg (*ts*13) of partially purified RNA polymerase were incubated at 33, 39.5, or 42°C. Samples (75 μl) were taken at the indicated times and spotted onto DEAE-cellulose filter paper disks. Symbols: ●, 33°C; ○, 39.5°C; △, 42°C.

the *ts*72 activity was markedly more thermolabile than the wild-type activity (Fig. 4A); the *ts*13 activity was somewhat more thermolabile than that of the wild type, while the *ts*8 activity was inactivated at the same rate as that of the wild type. Similar results were seen with several different polymerase preparations and when only a single ribonucleoside triphosphate was included in the preincubation mixture (data not shown). Increased thermolability was also observed when *ts*13 and *ts*72 activities were preincubated in the absence of all four ribonucleoside triphosphates (Fig. 4B) and when all three mutant activities were preincubated in the absence of both DNA and ribonucleoside triphosphates (Fig. 4C).

Evidence that *ts*72 contains two mutations within the RNA polymerase gene. Among the phenotypic characteristics of the mutants that have been examined so far, the strongest biochemical indication that the RNA polymerase of these mutants is altered is the increased thermolability of the *ts*72 polymerase activity. I considered the possibility that the thermolability of the *ts*72 polymerase activity could be the result of a second mutation that was distinct from that which is responsible for its *ts* replication in vivo. Therefore, I examined the thermolability of wild-type recombinants from a marker rescue experiment performed with the smallest cloned fragment of wild-type DNA that rescued *ts*72: a 2.85-kilobase-pair (kbp) fragment that extends from the *Hin*dIII-J and -H junction to the first *Eco*RI site on *Hin*dIII-H (16). When the marker rescue was performed under selective conditions at 39.5°C, I noted that although most of the plaques on the 39.5°C plaque assay plates were the same size as the wild-type plaques, 7% of the plaques were smaller than the wild-type plaques. However, if the marker rescue was performed under nonselective conditions (33°C), 25 to 30% of the recombinants that were capable of forming plaques at 39.5°C formed small plaques. Therefore, I plaque purified three large- and three small-plaque recombinants from independent marker rescue experiments and partially purified the RNA polymerase activity from purified virions as described above. The polymerase activities from all three of the large-plaque recombinants regained the thermolability pattern of the wild-type activity, while those from the small-plaque recombinants retained the thermolability of the *ts*72 activity. The data for one large- and one small-plaque recombinant are shown in Fig. 5.

The most likely explanation for these results is that *ts*72 does in fact contain two mutations, both of which lie within the 2.85-kbp *Hin*dIII-*Eco*RI fragment that was used in the marker rescue experiment. Although the major transcript encoded within the *Hin*dIII-H–*Eco*RI fragment is the 1.35K subunit of the RNA polymerase, one additional early and

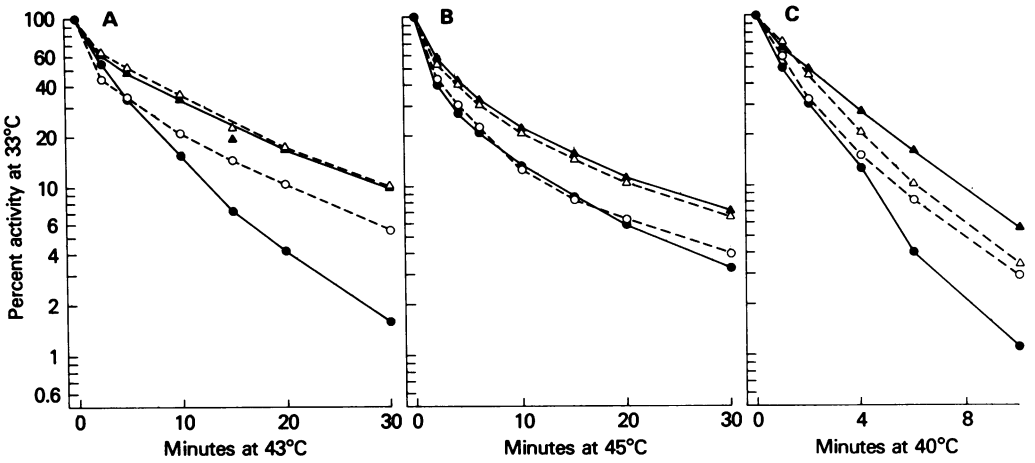

FIG. 4. Thermal inactivation of mutant and wild-type RNA polymerase activities. Partially purified RNA polymerase activities were incubated at the indicated temperatures in the standard RNA polymerase reaction mixture from which DNA (A), all four ribonucleoside triphosphates (B), or both DNA and ribonucleoside triphosphates (C) were omitted. Samples were withdrawn at the indicated times and assayed for residual polymerase activity at 33°C, as described in the text. Symbols: ▲, wild-type; △, *ts*8; ○, *ts*13; ●, *ts*72. The protein concentrations during the preincubation were as follows (in micrograms per milliliter): wild type, 11.3; *ts*8, 11.8; *ts*13, 22.8; *ts*72, 21.8.

two late transcripts have been mapped near the *Eco*RI site (5, 26, 27) (Fig. 6). To determine if both mutations lie within the gene for the RNA polymerase subunit, I employed two smaller subfragments of *Hin*dIII-H to rescue *ts*72: a 2,032-bp subfragment that extends from the *Hin*dIII-J and -H restriction site to a unique *Aha*III site and a slightly smaller fragment from the *Hin*dIII site to an *Ava*II site at nucleotide 1864 (Fig. 6). The *Hin*dIII-H–*Aha*III fragment contains all of the coding sequence of the RNA polymerase subunit that lies within *Hin*dIII-H and an additional 37 bp that includes 34 bp of coding sequence of a late 19K polypeptide (5). The *Ava*II site is 131 bp upstream of the polymerase subunit termination codon. Marker rescue was performed at 33°C, and the final yield of virus was assayed at 33 and 39.5°C to determine

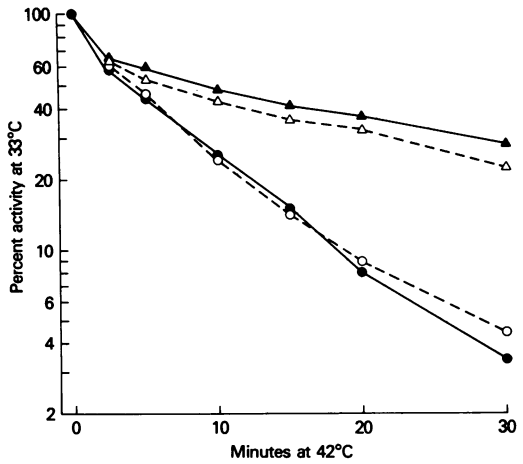

FIG. 5. Thermal inactivation of *ts*72 and recombinant RNA polymerase activities. Recombinants that were able to form plaques at 39.5°C were selected from marker rescue of *ts*72 with the wild-type 2.85-kbp *Hin*dIII-H–*Eco*RI DNA fragment (15). The partially purified RNA polymerase activities of one small-plaque and one large-plaque recombinant were preincubated at 42°C in the absence of DNA as described in the text. Symbols: ▲, wild type (11.9 μg/ml); △, large-plaque recombinant (8.8 μg/ml); ●, *ts*72 (15 μg/ml); ○, small-plaque recombinant (16.4 μg/ml).

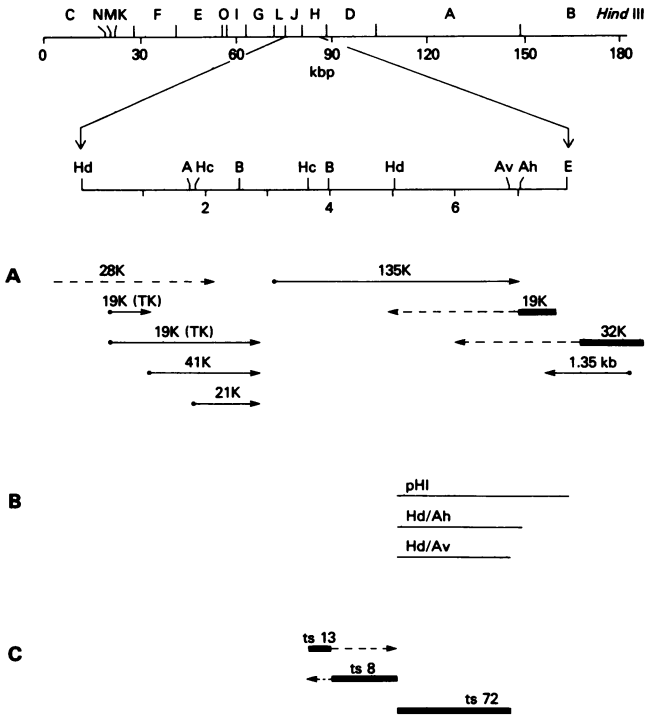

FIG. 6. Summary of restriction endonuclease sites, RNA transcripts, DNA fragments used in marker rescue, and physical map locations of *ts* mutants. Restriction endonuclease sites are taken from previously published reports (1, 3, 5, 26, 27, 34). Restriction endonucleases are abbreviated as follows: A, *Ava*I; Ah, *Aha*III; Av, *Ava*II, B, *Bgl*II; E, *Eco*RI; Hc, *Hin*cII; Hd, *Hin*dIII. (A) The positions of RNA transcripts are taken from previously published reports (1, 5, 20, 26, 27). The direction of transcription and size of the polypeptide product are shown. TK denotes the mRNA for the viral encoded thymidine kinase. Late mRNAs are diagrammed as solid lines, representing the minimum size required to encode the observed polypeptide product, followed by a dashed line to indicate the extent of 3′ heterogeneity (27). (B) Recombinant DNA fragments used in marker rescue of *ts*72 (Table 3) are shown. (C) The limits of the physical map positions of *ts* mutations are shown (16) (Table 2).

TABLE 3. Marker rescue of *ts*72

Expt no. and DNA[b]	Yield (PFU/ml) at[a]:				Efficiency of rescue yield (large or small plaques) at 39.5°C/yield at 33°C	
	33°C	39.5°C				
		Large plaques	Small plaques	% of total plaques that are large	Large plaque	Small plaque
1						
*Hin*dIII-H	6.0×10^7	7.6×10^5	3.0×10^5	72	1.3×10^{-2}	5.0×10^{-3}
*Hin*dIII-H	8.7×10^7	1.0×10^6	4.3×10^5	73	1.3×10^{-2}	4.9×10^{-3}
*Hin*dIII-H–*Aha*III	1.1×10^8	6.7×10^5	1.1×10^6	37	6.1×10^{-3}	1.0×10^{-2}
*Hin*dIII-H–*Aha*III	8.2×10^7	5.7×10^5	1.4×10^6	29	7.0×10^{-3}	1.7×10^{-2}
SS[c]	1.2×10^8	<70	<70		$<5.8 \times 10^{-7}$	$<5.8 \times 10^{-7}$
SS	6.8×10^7	<70	<70		$<1.0 \times 10^{-6}$	$<1.0 \times 10^{-6}$
2						
*Hin*dIII-H	2.2×10^8	1.3×10^6	3.9×10^5	76	5.9×10^{-3}	1.8×10^{-3}
*Hin*dIII-H	1.1×10^8	8.5×10^5	2.5×10^5	77	7.7×10^{-3}	2.3×10^{-3}
*Hin*dIII-H–*Ava*II	1.7×10^8	7.9×10^3	3.8×10^5	2	4.6×10^{-5}	2.2×10^{-3}
*Hin*dIII-H–*Ava*II	1.3×10^8	1.2×10^4	8.8×10^5	1.3	9.2×10^{-5}	6.8×10^{-3}
SS	1.2×10^8	<70	<70		$<5.8 \times 10^{-7}$	$<5.8 \times 10^{-7}$
SS	1.6×10^8	<70	<70		$<4.4 \times 10^{-7}$	$<4.4 \times 10^{-7}$

[a] Marker rescue was performed as a yield experiment in which cells infected with *ts*72 were transfected with the indicated DNAs and incubated at 33°C for 72 h.
[b] The *Hin*dIII-H fragment was cloned in pBR322 (3), and the *Hin*dIII-H–*Aha*III and *Hin*dIII-H–*Ava*II fragments were isolated by agarose gel electrophoresis after cleavage of the cloned *Hin*d-H fragment with the appropriate enzymes.
[c] SS, Salmon sperm DNA.

the proportion of large- and small-plaque recombinants. The yield of small-plaque recombinants was similar whether the entire *Hin*dIII-H fragment, the *Hin*dIII-H–*Aha*III fragment, or the *Hin*dIII-H–*Ava*II fragment was used for marker rescue (Table 3). However, the yield of large-plaque recombinants decreased dramatically when the smaller DNA fragments were employed: large-plaque recombinants represented over 70% of the progeny that were capable of forming plaques at 39.5°C when the *Hin*dIII-H fragment was used but only 29 to 37% or 1 to 2% when the *Hin*dIII-H–*Aha*III or the *Hin*dIII-H–*Ava*II fragments were used, respectively. Nevertheless, these results demonstrate that both mutations lie within the gene for the RNA polymerase subunit.

DISCUSSION

I mapped the *ts* mutations of three mutants of vaccinia virus to the gene that encodes one of the 135K subunits of the virion-associated DNA-dependent RNA polymerase (16) (Table 3 and Fig. 6). The requirement for this enzyme in the initiation of the vaccinia virus replication cycle has led to the prediction that *ts* mutants with defects in the RNA polymerase would not induce the synthesis of early viral proteins or replicate viral DNA at the nonpermissive temperature. However, none of the mutants described here had the predicted phenotype: all three induced the synthesis of early viral proteins and replicated viral DNA (1) (Fig. 1 and 2). Furthermore, the synthesis of RNA in vitro by permeabilized mutant virions was not *ts* relative to the wild type (Table 1). Only one mutant (*ts*13) synthesized an RNA polymerase activity at 33°C that displayed altered biochemical properties in vitro that could be associated with its *ts* mutation. The partially purified *ts*13 activity had a reduced specific activity, increased temperature sensitivity at 39.5 and 42°C, and increased thermolability under a variety of preincubation conditions. Although the *ts*72 activity was also more thermolabile than the wild-type activity, the thermolability marker was distinct from the *ts* mutation (see below). The lack of temperature sensitivity of the partially purified *ts*8 and *ts*72 activities could be explained if the *ts* defects of these mutants affected only accurate initiation on double-stranded vaccinia virus DNA because the partially purified enzyme initiates randomly on single-stranded DNA templates. However, the observation that early viral proteins and DNA are synthesized in cells that are infected by these mutants at 39.5°C and that even late viral proteins are synthesized in *ts*8-infected cells (and *ts*13-infected cells) demonstrates that the mutant RNA polymerases, once they are formed at 33°C, are able to initiate tanscription accurately at 39.5°C.

The most likely explanation for the results of this study is that these mutants are defective in the assembly of newly synthesized polymerase subunits into active enzyme or the incorporation of RNA polymerase into maturing virions. Once assembled at the permissive temperature, the activity may be stabilized by the association between different subunits or between the polymerase and other proteins or DNA within the virion. In the *Escherichia coli* system, the majority of *ts* mutations in the β and β′ subunits of the RNA polymerase (18) as well as some mutations in the α subunit (21) result in defective subunit assembly, and the polymerase activities synthesized at the permissive temperature are not more *ts* or thermolabile in vitro than is the wild type. These results point out the difficulty in identifying the biochemical defects of mutants with *ts* lesions that affect polypeptides that function as part of a multisubunit complex, particularly one as large and as complex as the vaccinia virus RNA polymerase.

During the course of this investigation, *ts*72 was found to contain two (or possibly more) mutations within the gene for the RNA polymerase subunit. In marker rescue experiments with subfragments of *Hin*dIII-H, two types of recombinants were observed: those that formed large plaques at 39.5°C and that contained an RNA polymerase with the thermolability pattern of the wild type and those that formed small plaques at 39.5°C and contained a polymerase with the thermolability of *ts*72. In one-step growth experiments at 39.5°C, cells infected with the small-plaque recombinants produced approximately 30 to 50% as much virus as cells infected with the wild type or with large-plaque recombi-

nants (data not shown). These results suggest that *ts*72 contains two mutations, one of which is primarily responsible for its *ts* replication and the second of which is responsible for the increased thermolability of the RNA polymerase. The small-plaque recombinants have acquired the wild-type allele at the *ts* locus, while the large-plaque recombinants are wild type at both the *ts* and thermolability loci. I did not attempt to isolate recombinants that were still *ts* but that had regained a thermostable polymerase activity. The greatly reduced proportion of large-plaque recombinants that were obtained in marker rescue experiments that employed DNA fragments that were shortened near the 3′ terminus of the mRNA (Table 3 and Fig. 6) suggests that the thermolability marker lies closer to the 3′ end of the gene than does the temperature sensitivity marker. It should be noted that *ts*72 was isolated after mutagenesis with nitrosoguanidine, which commonly induces multiple, closely spaced mutations (19).

The three mutants in this complementatin group differ in their ability to induce the synthesis of late viral polypeptides at the nonpermissive temperature. Late proteins are synthesized, although with some delay, in cells infected with *ts*8 or *ts*13, while the synthesis of late species is greatly reduced in *ts*72-infected cells. At present it is not known whether late genes are transcribed by the RNA polymerase of the infecting virion or by newly synthesized polymerase. Although results of early studies on the induction of the RNA polymerase indicated that it was a late function that appeared only after the onset of DNA replication, these studies were performed before conditions had been established that allowed the measurement of soluble polymerase activity (23, 31, 33). Thus, it is most likely that in these studies the particulate activity associated with maturing virions was measured. Because the mRNA for the 135K subunit is an early transcript and because it has been suggested that two early core polypeptides are in fact the high-molecular-weight subunits of RNA polymerase, it is possible that there is a small amount of newly synthesized RNA polymerase activity that is responsible for late transcription. If this is the case, the mutations in the *ts*8 and *ts*13 activities, but not in the *ts*72 activity, would have to allow sufficient polymerase to assemble to carry out late transcription. Alternatively, if the polymerase of the infecting virions transcribes late genes, it is possible that the *ts*72 activity is either unable to recognize late promoters or to interact with putative viral or host transcriptional factors that allow discrimination between early and late promoters. These three mutants may prove to be useful in deciphering the mechanism of the switch from early to late transcription.

ACKNOWLEDGMENTS

I thank S. Broyles and B. Moss for providing the DNA fragments that were used in marker rescue and for the sequence information regarding this region of the DNA before publication. I also thank B. Moss, R. Zandomeni, and K. Kahlili for helpful discussions and Michael Rovinsky for excellent technical assistance.

This study was supported in part by Public Health Service grant AI-18365 from the National Institutes of Health.

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