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DNA Synthesis and DNA Polymerase Activity of Herpes Simplex Virus Type 1 Temperature-Sensitive Mutants

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Fifteen temperature-sensitive mutants of herpes simplex virus type 1 were studied with regard to the relationship between their ability to synthesize viral DNA and to induce viral DNA polymerase (DP) activity at permissive (34 C) and nonpermissive (39 C) temperatures. At 34 C, all mutants synthesized viral DNA, while at 39 C four mutants demonstrated a DNA^+ phenotype, three were $DNA^{\pm}$, and eight were DNA^-. DNA^+ mutants induced levels of DP activity similar to those of the wild-type virus at both temperatures, and $DNA^{\pm}$ mutants induced reduced levels of DP activity at 39 C but not at 34 C. Among the DNA^- mutants, three were DP^+, two were $DP^{\pm}$, and three showed reduced DP activity at 34 C with no DP activity at 39 C. DNA^-, DP^- mutants induced the synthesis of a temperature-sensitive DP as determined by in vivo studies.

In the herpes simplex virus (HSV) replicative cycle, little is known concerning the number and function of genes involved in viral DNA synthesis. After infection with HSV, however, the levels of activity of several enzymes involved in nucleic acid metabolism have been shown to increase. These enzymes include thymidine kinase (12), DNA polymerase (6, 8, 9, 13), DNase (9), dTMP kinase (18), dCMP deaminase (8), and deoxycytidine kinase (6, 19).

It has been reported that the increase in DNA polymerase activity which occurs after infection with HSV is due to the appearance of a new DNA polymerase, the synthesis of which is controlled or specified by the viral genome (9-11, 16, 17, 27). Through the use of temperature-sensitive (ts) mutants exhibiting defects in DNA polymerase activity and/or viral DNA synthesis at the nonpermissive temperature, additional information may be obtained regarding both the process of viral DNA synthesis and the origin and role of DNA polymerase in HSV replication.

The present paper describes the relationship between the synthesis of viral DNA and the induction of DNA polymerase by 15 ts mutants of HSV-1. The results of these studies indicate that at least six cistrons control the synthesis and/or function of viral DNA and DNA polymerase. The finding that DNA^- mutants representing two distinct cistrons produce DNA polymerase molecules which are temperature sensitive in vivo lends further support to the concept that HSV codes for a new DNA polymerase(s) essential for the replication of viral DNA.

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MATERIALS AND METHODS

Cell cultures and media. Human embryonic lung (HEL) cells in 16-ounce prescription bottles and tubes were propagated in Eagle medium supplemented with 10% fetal bovine serum and 0.075% $NaHCO_3$ as previously described (23). Cultures were maintained in Eagle medium containing 5% fetal bovine serum and 0.15% $NaHCO_3$. Cells in petri dishes were propagated and maintained in Eagle medium supplemented with 10 and 5% fetal bovine serum, respectively, containing 0.225% $NaHCO_3$ in a 5% CO_2 atmosphere.

Viruses and virus assays. The KOS strain of type 1 HSV (HSV-1) was used as the wild-type (WT) virus, and permissive and nonpermissive temperatures were 34 and 39 C, respectively. The isolation, preliminary characterization, and nomenclature of ts mutants induced by 5-bromodeoxyuridine, nitrosoguanidine, and UV light have been described previously (21, 24). Virus stocks were prepared in HEL cell cultures at 34 C (21), and virus assays were performed by a plaque method utilizing a 2% methyl cellulose overlay (3). Constant-temperature water baths (±0.1 C) were used for incubation of closed vessels and water-jacketed CO_2 (5%) incubators (±0.2 C) were used for petri dishes.

Infection and labeling of cells for DNA determination. HEL cells in 8-ounce prescription bottles containing 3×10^6 to 5×10^6 cells were infected at a multiplicity of 5 to 10 PFU/cell with each mutant and the WT virus in duplicate experiments, each including all viruses to be tested. Multiplicities of infection were verified by simultaneous assays of

inocula. After incubation in maintenance medium at either 34 or 39 C for 6 h (to permit inhibition of host-cell DNA synthesis), medium was decanted, monolayers were washed twice with Tris-phosphate buffer at pH 7.4, and 10 ml of maintenance medium containing 10 μCi of [^{3}H]thymidine per ml (13 Ci/mM, Schwarz BioResearch Inc., Orangeburg, N.Y.) was added to each culture. Infected cells were harvested 24 h after infection by scraping into medium. One milliliter of uniform cell suspension was removed and frozen at -90 C. This sample was later thawed, sonicated at 10 kc for 40 s, and centrifuged at 10^3 rpm for 15 min at 5 C. The supernatant fluid was assayed immediately for infectivity at 34 and 39 C in HEL cell monolayers.

Separation of viral and cellular DNA. Cells in the remaining 9 ml of suspension were pelleted, washed once in 10 ml of TNE (0.01 M Tris-hydrochloride, 0.1 M NaCl, and 0.001 M EDTA, pH 7.4) and repelleted. TNE was decanted, tubes were drained, and pellets were frozen at -90 C. Cell pellets were later thawed, resuspended in 3.5 ml of TNE, and lysed by the addition of 0.18 ml of 10% sodium lauryl sarcosinate and 0.07 ml of 10% sodium dodecyl sulfate at final concentrations of 0.5 and 0.2%, respectively. Suspensions were incubated at room temperature for 10 min, Pronase (Calbiochem, La Jolla, Calif.) was added to a final concentration of 50 μg/ml, and suspensions were incubated at 37 C for 30 min. One milliliter of each lysate and 9 ml of TNE were mixed and the refractive index of the solution was adjusted to 1.4010 in CsCl. Samples were centrifuged in CsCl at 143,000 $\times$ g for 68 h using a Beckman 75 Ti-rotor (Beckman Instruments, Palo Alto, Calif.) and a Beckman L2-75B ultracentrifuge at 25 C. After centrifugation, 40 0.3-ml fractions were collected (Isco density gradient fractionator, model 640; Instrumentation Specialties Co., Lincoln, Neb.) and 50 μl of each fraction was placed on Whatman GF/A filter disks. Disks were dried, washed once in 5% trichloroacetic acid (4 C), once in water (4 C), redried, and placed in scintillation vials containing 7 ml of counting solution containing toluene and BioSolv (Beckman). Samples were counted using a Beckman LS250 liquid scintillation counter.

Counts per fraction were plotted and the percentage of both cellular and viral DNA in mutant-infected cell extracts was determined using a DuPont model 310 curve resolver.

Induction of DNA polymerase (DP) activity in infected cells: (i) infection of monolayers. HEL cells were seeded in 100-mm petri plates, 3 $\times$ 10^6 cells/plate. Twenty-four to forty-eight h later, when monolayers were confluent, duplicate cultures were infected at a multiplicity of infection of 5 to 10 PFU/cell. After adsorption of virus for 1 h at 37 C, the inoculum was decanted, monolayers were washed twice with Tris, and 10 ml of maintenance medium was added to each culture. Cultures were further incubated for 3 to 13 h at 34 or 39 C and infected cells were harvested by trypsinization. Cells were pelleted by low-speed centrifugation (approximately 10 $\times$ 10^6 to 16 $\times$ 10^6 cells/pellet), washed with Tris, and repelleted. Mock-infected control cultures were treated similarly. For determination of infectivity to parallel kinetic studies of DP activity, 2 $\times$ 10^5 HEL cells in tubes were infected as described for induction of enzymes. Cultures were harvested at the indicated times by one cycle of freezing and thawing followed by 45 s of sonication at 4 C with a Raytheon sonic oscillator. Suspensions were clarified by low-speed centrifugation and supernatant fluids were assayed at 34 C in HEL cell monolayers.

(ii) Preparation of enzyme extracts. Infected and control cell pellets were resuspended in 5 to 10 volumes of hypotonic enzyme extraction solution consisting of 0.003 M 2-mercaptoethanol and 0.01 M Tris buffer, pH 7.5. Clarified whole-cell enzyme extracts were obtained by sonication at 4 C, 10 kc for 3 min followed by centrifugation for 1 h at 30,000 $\times$ g at 4 C. The protein content of the enzyme extracts was determined by the method of Lowry et al. (15).

(iii) Enzyme assays. DP activity was assayed by the method of Kit et al. (14), modified to include 100 mM $(NH_4)_2SO_4$ for infected cell enzyme extracts (11). Briefly, incubation mixtures contained 20 mM sodium phosphate, pH 7.5, 8 mM $MgCl_2$, 6.6 mM Tris-hydrochloride, pH 7.5, 2 mM 2-mercaptoethanol, 100 μg of heat-denatured salmon sperm DNA, 0.266 mM thymidine-5'-[*methyl*-^{3}H]triphosphate ([^{3}H]-TTP; 48 Ci/mM; New England Nuclear Corp., Boston, Mass.), and 0.266 mM each of dATP, dCTP, and dGTP (P. L. Biochemicals, Inc., Milwaukee, Wis.), in a total volume of 200 μl. The standard assay time was 30 min at 39 C. Under the conditions of the assay there was a linear dose response between the amount of [^{3}H]TTP incorporated with time (for 60 min or more), and with the amount of enzyme added per tube (80 to 300 μg of protein). The reaction was stopped by the addition of cold 5% trichloroacetic acid and the incorporated nucleotides (trichloroacetic acid-insoluble fractions) were processed by washing three times in 5% trichloroacetic acid followed by solubilization with 1 ml of NCS (Amersham/Searle, Arlington Heights, Ill.). Radioactivity was determined in a Beckman LS-250 liquid scintillation counter.

Heat inactivation of polymerase. In vitro heat inactivation of enzyme samples was performed as follows: 100 μl of enzyme containing approximately 200 μg of protein in stoppered tubes was placed in a constant temperature water bath ($\pm$0.1 C) at 39 C. At the indicated times, duplicate tubes were withdrawn and placed in an ice bath. DNA polymerase activity was then determined using 50-μl aliquots of the heated enzyme preparation. In vivo heat inactivation of DP activity was determined by temperature shift-up experiments. Cultures infected as described above were maintained at 34 C for 11 h followed by shift-up to 39 C. Cultures were harvested at the indicated times after shift-up and assayed for DP activity as described above.

RESULTS

Synthesis of viral and cellular DNA by WT virus and ts mutants. Fifteen ts mutants of HSV-1, belonging to 10 complementation groups (21), were used in this study. In an effort

to determine whether the ts function exhibited by each of the 15 mutants had an effect on viral and cellular DNA synthesis, the incorporation of [^{3}H]thymidine into DNA in mutant- and WT virus-infected cells at 34 and 39 C was examined. Although the viral DNA phenotypes of the mutants at 39 C had been determined previously by analytical ultracentrifugation of infected cell extracts (21), preparative centrifugation of isotopically labeled extracts was carried out since this technique offers a more sensitive method for the quantification of viral and cellular DNA synthesis.

Characteristic patterns of incorporation of [^{3}H]thymidine into viral and cellular DNA by the WT virus and five representative mutants in a single, simultaneous experiment are shown in Fig. 1. Mock-infected cells gave a single peak of cellular DNA with a buoyant density of 1.6900 to 1.700 g/cm^{3} (Fig. 1). WT virus-infected cells at both 34 and 39 C, all mutant-infected cells at 34 C and some at 39 C (e.g., tsF18 and tsG8), gave two peaks, one corresponding to cellular DNA and one, with a buoyant density of 1.725 g/cm^{3}, corresponding to viral DNA. No difference in buoyant density was observed between the viral DNAs of the mutants and that of the WT virus.

The average values for viral and cellular DNA synthesis in two or three tests with all 15 mutants and the WT virus are shown in Table 1. Although all mutants produced viral DNA at 34 C, only three of them (tsN20, tsG8, and ts022) did so as efficiently as the WT virus. Other mutants synthesized from 12 (tsC4) to 75% (tsB2) as much viral DNA as the WT virus. Reduced amounts of [^{3}H]thymidine-labeled viral DNA were synthesized by WT virus-infected cells at 39 C as compared with 34 C (Fig. 1). Compared with the WT virus at 39 C, four mutants (tsE6, tsF18, tsI11, and tsN20) in four complementation groups synthesized 50% or more as much viral DNA (DNA^{+} mutants), and three mutants (tsG3 [16%], tsG8 [3%], and tsO22 [4%]) in two complementation groups synthesized only minimal amounts of viral DNA at 39 C (DNA$^{\pm}$ mutants). Eight mutants belonging to four complementation groups failed to synthesize detectable amounts of viral DNA at this temperature (DNA^{-} mutants). The quantities of viral DNA synthesized by mutants belonging to the same complementation group at 34 C were roughly similar. Likewise, at 39 C the viral DNA phenotypes of mutants in groups with multiple members (i.e., groups A, B, C, and G) were similar.

With regard to cellular DNA synthesis, the WT virus inhibited cellular DNA synthesis to 36% of mock-infected cell levels at 34 C (Fig. 1, Table 1). Fourteen of the fifteen mutants exhibited even greater inhibition of cellular DNA synthesis at 34 C than the WT virus. In extracts of cells infected with these mutants, residual cellular DNA synthesis ranged from 10 (tsI11) to 30% (tsB2) of mock-infected cell levels. One mutant, tsG3, however, was about half as efficient as the WT virus in inhibiting cellular DNA synthesis in that 60% of the DNA synthesized in mock-infected cells was synthesized during the 6- to 24-h labeling period. At 39 C the WT virus and all 15 mutants inhibited cellular DNA synthesis as efficiently or more efficiently than at 34 C (Table 1). At 39 C, 22% of the mock-infected level of cellular DNA was synthesized in WT virus-infected cells, representing 78% inhibition. Six mutants (tsE6, tsF18, tsN20, tsA15, tsB2, and tsB21) inhibited cellular DNA synthesis as efficiently as the WT virus at this temperature while the remaining nine mutants were more efficient inhibitors in that residual levels of cellular DNA ranged from 4 (tsA1 and tsG8) to 14% (tsG3 and tsC4) of

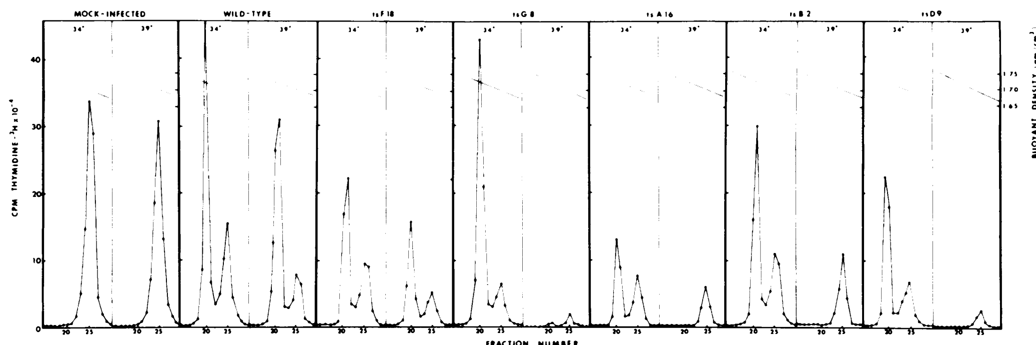

FIG. 1. *Separation of viral and cellular DNAs by CsCl equilibrium centrifugation. Mock-infected WT virus and mutant-infected HEL cells incubated at 34 and 39 C were exposed to 10 μCi of [^{3}H]thymidine per ml from 6 to 24 h pi. Lysates of these cells were then subjected to equilibrium centrifugation in neutral CsCl gradients.*

TABLE 1. *Viral and cellular DNA synthesis of WT virus and ts mutants of HSV-1 at 34 and 39 C*

Virus	Viral DNA[a, c]		Cellular DNA[b, c]	
	34 C	39 C	34 C	39 C
WT	100	100	36	22
Mock-infected cells			100	100
tsE6	72	68	21	23
tsF18	63	50	28	17
tsI11	59	64	10	8
tsN20	94	82	24	21
tsG3	57	16	60	14
tsG8	96	3	18	4
tsO22	99	2	21	6
tsA1	44	0	11	4
tsA15	19	0	24	20
tsA16	33	0	21	16
tsB2	75	0	30	29
tsB21	56	0	24	21
tsC7	27	0	18	11
tsC4	12	0	15	14
tsD9	57	0	23	7

[a] Results are expressed as the percentage of viral DNA in WT virus-infected cultures.

[b] Results are expressed as the percentage of cellular DNA in mock-infected cultures.

[c] Values given represent the average of two or three separate experiments.

levels in mock-infected control cells (Table 1).

At 39 C no significant correlation was observed between the viral DNA synthetic capabilities of the mutants and their ability to inhibit cellular DNA synthesis. Mutants which inhibited cellular DNA synthesis as efficiently as the WT virus at 39 C synthesized either large amounts (e.g., tsN20), intermediate amounts (e.g., tsF18), or no (e.g., tsA15, tsB2, and tsB21) viral DNA at 39 C as compared with the WT virus at this temperature (Table 1).

Induction of DP activity by WT virus and ts mutants. In order to establish optimum conditions for assay of HSV-1 DP activity, the effects of $(NH_4)_2SO_4$ concentration, temperature of assay, and time of assay were first examined.

As previously shown by Keir et al. (11), infected cell extracts were optimally active at 100 mM $(NH_4)_2SO_4$, whereas uninfected cell extracts were optimally active in the absence of $(NH_4)_2SO_4$ and significantly less active at 100 mM (Fig. 2). Therefore, in subsequent assays of WT virus and ts mutant-induced polymerase activity, optimal conditions of NH_4^+ ion concentration (i.e., 100 mM) were employed.

In order to determine the effect of permissive and nonpermissive growth temperature on DP activity, enzyme extracts from both WT virus-infected and uninfected control cells, grown for 10 h at 34 C, were assayed at 34 and 39 C. The results demonstrate that DP activity from both infected and uninfected cells was approximately twofold greater when assayed at 39 C as compared to 34 C (Fig. 3). Initial screening of mutant-infected cell extracts for DP activity, therefore, was carried out at 39 C for 30 min. In addition, failure to detect DP activity in initial screening tests performed at 39 C would suggest that the enzyme itself might be temperature-sensitive.

To determine the time of maximal DP activity during the HSV replicative cycle at both permissive and nonpermissive temperatures, the kinetics of DP activity were studied in WT virus-infected HEL cells and in mock-infected cells sampled at intervals during the HSV replicative cycle (Fig. 4). When DP assays were performed in the absence of NH_4^+ ion, with extracts of infected cells grown at either 34 or 39 C, a 1.5- to 2-fold increase in activity above

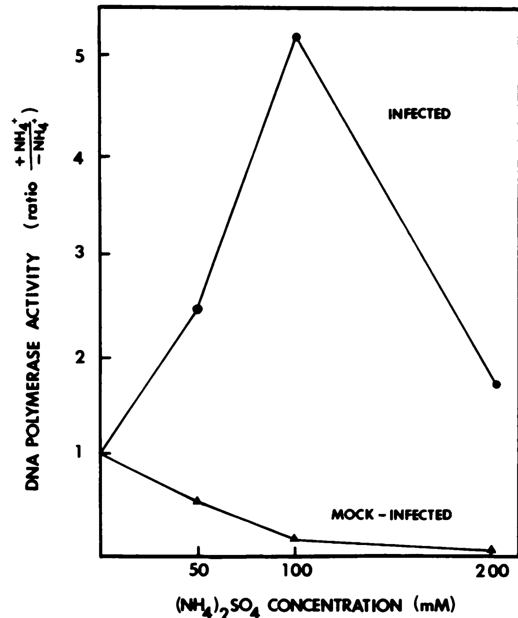

FIG. 2. *Effect of NH_4^+ concentration on DP activity. Mock-infected and infected cells were harvested after 10 h of incubation at 34 C. DP activity was assayed in the presence of the indicated concentrations of $(NH_4)_2SO_4$ for 30 min at 39 C. DP activity is expressed as the ratio of picomoles of TTP incorporated per microgram of protein in the presence of $(NH_4)_2SO_4$ to the incorporation in the absence of $(NH_4)_2SO_4$. Symbols: (●) infected cultures and (▲) mock-infected cultures.*

that of uninfected control cell activity was observed from 4 to 14 h postinfection (pi). Assays performed with 34 C-infected cell extracts in the presence of NH_4^+ ion, however, demonstrated a marked increase in DP activity beginning at 4 h pi and reaching maximum levels at 10 to 12 h pi. This increase represented an approximate 12-fold increase in polymerase activity when compared with the activity observed in uninfected cells. Under these conditions an increase in infectious virus was first apparent at 6 h pi and had not reached maximum levels by 14 h. DP activity assayed with NH_4^+ ion from extracts of infected cells grown at 39 C also began to increase by 4 h pi and reached a maximum at 9 to 10 h pi. This increase represented a sixfold increase in activity above uninfected control cell levels. Under these conditions an increase in infectious virus was apparent after 4 h and maximum titers were reached from 12 to 14 h pi. Ten hours pi was therefore selected as the time of assay for DP activity of ts mutant-infected cells grown at 34 and 39 C.

DP activity of HSV-1 ts mutants. The DP activity induced by the WT virus and 15 HSV-1 ts mutants after 10 h of incubation at 34 and 39 C is shown in Table 2. At 34 C polymerase activity in mock-infected cultures was only 1% of that in WT virus-infected cultures, while all but three mutants (tsC4, tsC7, and tsD9) synthesized from 72 to 116% of WT levels of DP activity. At 39 C mutants belonging to complementation groups E, F, I, and N, which synthesized 50% or greater of the amount of viral DNA synthesized by the WT virus (Table 1), induced WT levels of DP activity (DNA⁺,

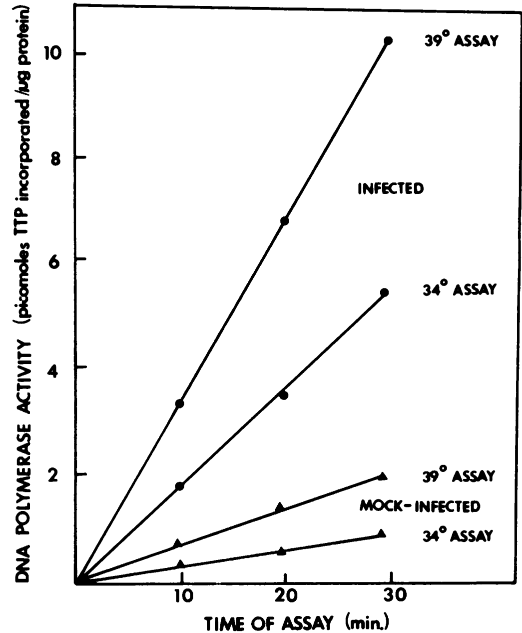

FIG. 3. *Effect of temperature of assay on DP activity. Mock-infected and infected cells were harvested after 10 h of incubation at 34 C. DP activity was assayed at either 34 or 39 C for 30 min at the indicated time pi in the presence of $(NH_4)_2SO_4$ (100 mM) for infected cells (●). Mock-infected cells (▲) were assayed in the absence of $(NH_4)_2SO_4$.*

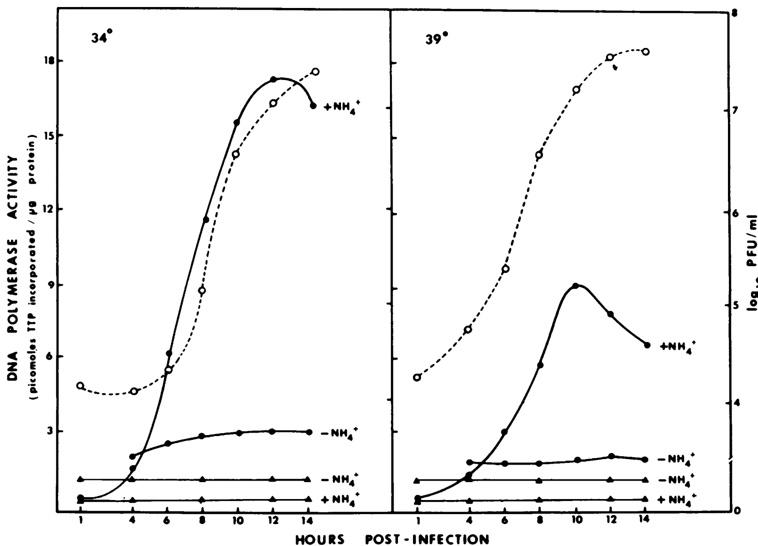

FIG. 4. *Kinetics of induction of DP activity. Mock-infected (▲) and infected (●) cells were incubated at either 34 or 39 C and harvested at the indicated times pi. Extracts were assayed for DP activity at 39 C for 30 min in the presence and absence of $(NH_4)_2SO_4$ (100 mM). Infectivity assays were performed to parallel kinetics of enzyme induction (O).*

TABLE 2. *Induction of DNA polymerase activity by WT virus and ts mutants of HSV-1 at 34 and 39 C*

Virus	DNA polymerase activity[a]		Phenotype at 39 C	
	34 C[b]	39 C	DNA polymerase	Viral DNA
WT	100[b]	100[b]		
Mock infected	1	3		
tsE6	73	141	+	+
tsF18	108	80	+	+
tsI11	112	82	+	+
tsN20	116	152	+	+
tsG3	86	20	±	±
tsG8	109	14	±	±
tsO22	81	5	±	±
tsA1	103	95	+	−
tsA15	72	114	+	−
tsA16	80	102	+	−
tsB2	95	24	±	−
tsB21	74	38	±	−
tsC4	35	1	−	−
tsC7	41	3	−	−
tsD9	34	1	−	−

[a] Cultures were infected at a multiplicity of 5 PFU/cell and incubated at either 34 or 39 C for 10 h. Mock-infected and infected cell extracts were prepared and DP activity was determined in the presence of 100 mM $(NH_4)_2SO_4$. Results are expressed as picomoles of TTP incorporated per microgram of protein in 30 min at 39 C relative to WT virus-infected cultures as 100%. Values given represent the average of two to four separate determinations.

[b] DNA polymerase activity: 100% equals 17.5 and 8.9 picomoles of TTP incorporated per microgram of protein/h at 34 C and 39 C, respectively.

DP^+ mutants). Mutants belonging to complementation groups G and O induced reduced amounts of both viral DNA (Table 1) and DP activity ($DNA^{\pm}$, $DP^{\pm}$ mutants). The remaining mutants studied, all of which were DNA^- at 39 C (Table 1), fell into three categories with respect to their ability to induce DP activity at 39 C (Table 2): (i) mutants belonging to complementation group A, which induced activity at levels comparable to that of the WT virus (DNA^-, DP^+ mutants), (ii) mutants in complementation group B, which induced only about one-third of the WT virus level of DP activity (DNA^-, $DP^{\pm}$ mutants), and (iii) mutants in complementation groups C and D, which induced DP activity equal to or less than that produced in mock-infected cells (DNA^-, DP^- mutants). Mutants in this group also induced less than 50% of the level of WT DP activity at 34 C. These results suggested that at 39 C mutants which were phenotypically $DP^{\pm}$ or DP^- either (i) contained an inhibitor of DP activity, (ii) produced a temperature-sensitive DNA polymerase molecule, or (iii) were defective in the induction of DP. When assays were conducted at 34 C, no increase in DP activity was detected with any of the mutants, indicating that none of the mutants produced DP at 39 C which was inactivated in the 39 C assay.

Inhibitor studies. The possibility that mutants in complementation group B grown at 39 C and mutants in complementation groups C and D grown at 34 and 39 C produced a freely diffusable inhibitor of DP resulting in reduced levels of enzyme activity was examined. Extracts of cells infected at 34 or 39 C with mutants in groups B, C, and D were mixed in equal amounts with extracts of cells infected with the WT virus and incubated at the same temperature (Table 3). In all cases the DP activity in mixed extracts was approximately equal to one-half the sum of the activities of each single extract in the mixture. The reduction in DP activity in mixed extracts corresponded to the dilution of the activity in the WT extract by the mutant extract and indicated that no freely diffusable inhibitor of DP activity was present in either group B, C, or D mutant-infected cells.

Heat inactivation of DP activity. Heat inactivation studies were performed with group B,

TABLE 3. *DNA polymerase activity in mixed extracts of WT virus and ts mutant-infected cells*[a]

Virus	TTP (pmol incorporated)			
	34 C		39 C	
	Single extracts	Mixed extracts	Single extracts	Mixed extracts
WT	16.1		9.2	
tsB2	15.7	15.9	2.7	6.0
tsB21	13.9	15.2	3.9	6.2
tsC4	4.8	10.0	0.1	4.5
tsC7	5.2	10.4	0.2	4.5
tsD9	4.6	9.8	0.1	4.7

[a] Cultures were infected at a multiplicity of 5 PFU/cell and incubated at either 34 or 39 C for 10 h. Equal volumes of single enzyme extracts were mixed with extracts of WT virus-infected cells grown at either 34 or 39 C. DP activity was assayed with 50 μl of single or mixed extracts, and results are expressed as picomoles of TTP incorporated per microgram of protein in 30 min at 39 C.

C, and D mutants in order to determine whether they induced DP which was temperature sensitive in vitro and/or in vivo.

For in vitro studies infected cell extracts were preheated for 10, 20, and 30 min at 39 C, and residual activity was compared with enzyme extracts of cells infected with WT virus and group A (DP^+ mutants) treated in the same manner. None of the mutants tested induced DP which was more heat labile than the WT enzyme (Fig. 5). Residual polymerase activity of both WT and mutant extracts ranged from 50 to 60% of initial activity after 30 min at 39 C.

For in vivo studies infected cells maintained at 34 C for 11 h were shifted to 39 C and residual DP activity was determined after 1, 2, and 3 h and compared with that of WT virus-infected cells treated in the same manner. One set of infected cultures was maintained at 34 C to determine normal enzyme levels at the permissive temperature. The results of in vivo heat inactivation of DP are shown in Fig. 6. DP activity in all cultures maintained at 34 C from 0 to 14 h pi remained constant (tsB2, WT, and tsC4) or increased (tsD9 and tsC7). While DP activity in WT and tsB2-infected cultures declined to about 80% of the original activity 3 h after shift-up to 39 C, activity in cultures infected with tsC4, tsC7, and tsD9 declined to less than 25% of the activity present at the time of shift. Thus, in vivo DP activity of mutants in groups C and D was significantly more heat-labile than that of the WT virus whereas that of the group B mutant was not.

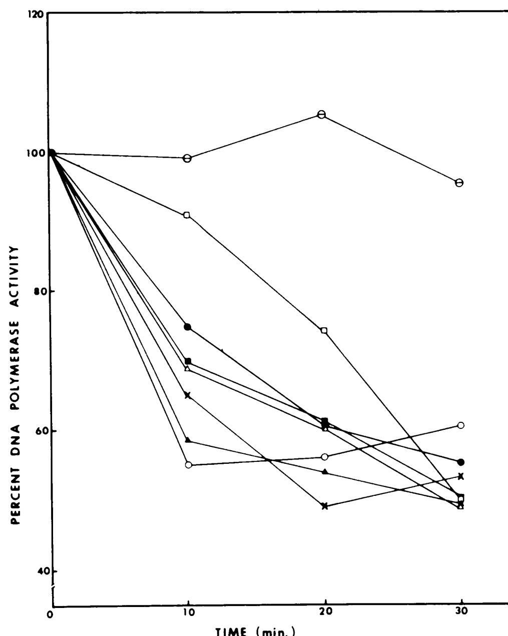

FIG. 5. *In vitro heat inactivation of WT virus and ts mutant-infected cell extracts. Infected cells were harvested after 10 h of incubation at 34 C. Enzyme extracts were heated at 39 C for the indicated times and assayed for DP activity in the presence of 100 mM* $(NH_4)_2SO_4$. *Symbols:* (⊖) *mock-infected,* (●) *WT,* (○) *tsB2,* (×) *tsD9,* (■) *tsA1,* (□) *tsA16,* (▲) *tsC4, and* (△) *tsC7-infected cell extracts. Results are presented relative to unheated extracts as 100%.*

DISCUSSION

In an effort to determine the biochemical nature of the temperature-sensitive defects exhibited by HSV-1 ts mutants, the relationship between their ability to synthesize viral DNA and to induce DP activity was examined at both permissive and nonpermissive temperatures.

The abilities of mutants to synthesize viral DNA and to inhibit cellular DNA synthesis were measured by preparative ultracentrifugation. Determination of viral DNA phenotypes by both analytical (21) and preparative ultracentrifugation yielded identical results. Unlike analytical ultracentrifugation, however, the preparative technique enables one to measure the synthesis of viral DNA as well as the inhibition of cellular DNA synthesis.

Of the eight mutants which were found to be viral DNA^-, all have been demonstrated to induce the synthesis of virus-specific proteins as detected by immunofluorescence and polyacrylamide gel electrophoresis (R. J. Courtney, personal communication), implying that their temperature-sensitive defects do not involve early functions such as adsorption, penetration, and uncoating. Therefore, the failure to detect [^{3}H]thymidine incorporation into viral DNA at 39 C by these mutants (representing four of 15 complementation groups so far identified [21]) suggests either that insufficient viral DNA was synthesized to allow detection by the methods employed or that no viral DNA was synthesized.

It is possible that the lateness of the 24-h harvest time may have underestimated the extent of viral DNA synthesis since virus particles released into the medium of infected cultures were not collected and added to the pool of cell-associated DNA. It should be noted, however, that the encapsidation of viral DNA into virions in herpesvirus infection is relatively inefficient (1). Thus, the majority of viral DNA synthesized would remain within the nucleus. Furthermore, not all mutants are able to synthesize physical particles at 39 C, and those which do synthesize primarily "empty" parti-

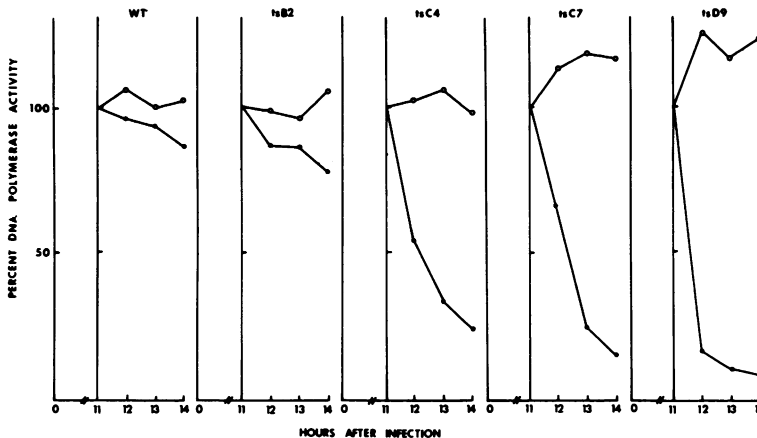

FIG. 6. *In vivo heat inactivation of WT virus and ts mutant-induced DP activity. Infected cells were incubated in duplicate at 34 C for 11 h when half the cultures were shifted to 39 C. Cultures were harvested at the indicated times pi and assayed for DP activity in the presence of 100 mM* $(NH_4)_2SO_4$*; (○) cultures maintained at 34 C (0 to 14 h pi); (●) cultures shifted to 39 C at 11 h pi. Results are presented relative to DP activity detected in cultures at 11 h, the time of shift, as 100%.*

cles (22) which would not deplete the viral DNA pool.

Although no conclusive relationship between the inhibition of host-cell DNA synthesis and the synthesis of viral DNA was demonstrated in the present study, the data of Pringle et al. (20), with ts mutants of pseudorabies virus, suggested a temporal relationship between these two virus-induced functions. Little is presently known about the mechanism involved in the inhibition of cellular DNA synthesis; however, the availability of ts mutants with altered capacities to inhibit cellular DNA synthesis will permit the examination of this problem in greater detail.

The results of these and other (G. M. Aron, P. A. Schaffer, and M. Benyesh-Melnick, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, V42, p. 201) studies indicate that a minimum of six HSV-1 cistrons (A, B, C, D, G, and O) are involved in either the production or regulation of viral DP and DNA synthesis. (i) Mutants in groups G and O ($DNA^{\pm}$, $DP^{\pm}$) induce reduced levels of DP and may be defective either in the production of DP or in the ability to synthesize a factor(s) regulating the activity of the enzyme. (ii) Mutants in groups A (DNA^{-}, DP^{+}) and B (DNA^{-}, $DP^{\pm}$) appear to be defective in regulating the ability of DP to function properly in vivo, since no viral DNA was synthesized in the presence of functional, thermostable DP. (iii) Mutants in complementation groups C and D (DNA^{-}, DP^{-}) appear to induce a temperature-sensitive DP as determined by in vivo studies, suggesting that the ts defects of these mutants are due to the production of a temperature-sensitive polymerase molecule per se.

The present study of the relationship between the ability of the ts mutants to induce DP and synthesize viral DNA indicate that HSV-DNA replication is not an essential requirement for the induction of DP. Thus, mutants belonging to complementation groups A and B induce DP and yet are unable to synthesize viral DNA. The data also suggest that viral DP is essential for DNA replication since (i) none of the mutants tested synthesized viral DNA in the absence of DP, and (ii) a correlation was observed between the amount of viral DNA synthesized and the level of DP activity found in the DNA^{+}, DP^{+} and $DNA^{\pm}$, $DP^{\pm}$ mutant groups. Although DP may be essential for DNA synthesis, it is not the sole requirement since complementation group A and B mutants induced DP yet were phenotypically DNA^{-}.

Additional evidence for the apparent involvement of a large number of cistrons (of an approximate total of 100 HSV genes) in the synthesis and/or regulation of viral DP and DNA comes from studies with other herpesvirus ts mutants. Three of eight cistrons of HSV-1 were found by Timbury and Subak-Sharpe (26) to affect viral DNA synthesis. Studies by Esparza et al. (4) also indicate that at least three of a total of seven cistrons so far identified are involved in the expression of the HSV-2 DP and DNA phenotypes. Mutants defective in two of these cistrons induce DP that is temperature-sensitive in vivo but not in vitro (Purifoy and Benyesh-Melnick, submitted for publication).

In studies of another series of HSV-2 ts mutants, Halliburton and Timbury (5) demonstrated that seven of the 10 cistrons affected the synthesis of viral DNA; two of the seven cistrons apparently affect DP induction and mutants defective in one of the two cistrons exhibit temperature-sensitive DP activity in vivo but not in vitro (J. Hay, personal communication). Pringle et al. (20) reported that members of four of the nine complementation groups of ts mutants of pseudorabies virus were unable to synthesize more than 1% of WT virus levels of viral DNA.

Immunological and biochemical studies (5) indicate that the HSV-induced polymerase is distinct from the host-cell enzyme and hence is virus coded. The finding that complementation group C and D ts mutants induce polymerase activity which is temperature sensitive in vivo represents further suggestive evidence that HSV codes for a new DP. Furthermore, since mutants belonging to two different complementation groups induced a temperature sensitive DP, there may be two subunits involved in the formation of a functional HSV-induced polymerase.

Confirmation of the hypothesis that HSV codes for a new polymerase must await the demonstration that the temperature-sensitive lesions reside in the induced molecule(s) as has been shown for bacteriophage T4 and T5 DP (2). The conversion of an inactive form of T4 DP upon temperature shift-down to a functional enzyme has been shown for a T4 bacteriophage mutant bearing a temperature-sensitive defect in the structural gene for DP (7, 25).

Experiments are in progress to determine whether the temperature-sensitive polymerase defect of group C and D HSV-1 ts mutants is involved in the initiation and/or elongation of viral DNA synthesis in vivo. More detailed studies of these and other mutants of HSV-1 should prove to be useful in elucidating the biochemical events involved in HSV DNA replication.

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