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Viral DNA Synthesis in Cells Infected with Temperature-Sensitive Mutants of Herpes Simplex Virus Type 1

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Temperature-sensitive mutants of herpes simplex virus type 1 representing eight DNA-negative complementation groups were grouped into the following three categories based on the viral DNA synthesis patterns after shift-up from the permissive to the nonpermissive temperature and after shift-down from the nonpermissive to the permissive temperature in the presence and absence of inhibitors of RNA and protein synthesis. (i) Viral DNA synthesis was inhibited after shift-up in cells infected with *ts*B, *ts*H, and *ts*J. After shift-down, *ts*B- and *ts*H-infected cells synthesized viral DNA in the absence of de novo RNA and protein synthesis whereas *ts*J-infected cells synthesized no viral DNA in the absence of protein synthesis. The B, H, and J proteins appear to be continuously required for the synthesis of viral DNA. (ii) Viral DNA synthesis continued after shift-up in cells infected with *ts*D and *ts*K whereas no viral DNA was synthesized after shift-down in the absence of RNA and protein synthesis. Mutants *ts*D and *ts*K appear to be defective in early regulatory functions. (iii) Cells infected with *ts*L, *ts*S, and *ts*U synthesized viral DNA after shift-up and after shift-down in the absence of RNA and protein synthesis. The functions of the L, S, and U proteins cannot yet be determined.

The temperature-sensitive (*ts*) mutants of herpes simplex virus type 1 (HSV-1) have been divided into two classes on the basis of whether or not viral DNA is synthesized upon continuous incubation at the nonpermissive temperature: mutants which do synthesize viral DNA are called DNA positive and those which do not are called DNA negative (DNA^-) (1, 8, 10; for reviews see 7, 11). Defects in several viral functions could result in a DNA^- phenotype: (i) a defective product continuously required for the elongation of viral DNA; (ii) a defect in a gene whose product is required for the initiation of viral DNA synthesis; and (iii) the temperature-sensitive expression of early, regulatory functions not directly involved in DNA synthesis but required for the transcription of genes whose products are needed for viral DNA synthesis. The general nature of the defect of DNA^- mutants can be determined by measuring viral DNA synthesis after temperature shifts of mutant-infected cells. If a protein continuously required for viral DNA synthesis was temperature sensitive, then viral DNA synthesis would be inhibited upon a shift from the permissive to the nonpermissive temperature. If an early, regulatory viral function was temperature sensitive, the viral DNA synthesis would continue after shift-up. Schaffer et al. (9), using their mutants in four DNA^- complementation groups, found that mutants in three groups synthesized little or no viral DNA after shift-up to the nonpermissive temperature whereas a mutant in the fourth group synthesized nearly normal amounts of viral DNA after shift-up. The objective of the present study was to characterize the defect in viral DNA synthesis in cells infected with mutants in eight DNA^- complementation groups (1, 5, 10) by use of temperature-shift experiments. Viral DNA synthesis was measured in mutant-infected cells (i) after shift-up to the nonpermissive temperature to determine whether synthesis continued or was inhibited and (ii) after shift-down to the permissive temperature in the presence and absence of inhibitors of protein and RNA synthesis. Early regulatory mutants would be expected to require de novo RNA and protein synthesis after temperature shift-down whereas mutants defective in functions directly involved in DNA synthesis might not, depending on the stability of the gene product under nonpermissive conditions.

MATERIALS AND METHODS

Cells and virus. BHK-21 clone 13 cells (4) were grown in Eagle medium (Dulbecco modification) with 10% (vol/vol) calf serum (Colorado Serum Co.) and 10% (vol/vol) tryptose phosphate broth (Difco) on 3-

cm plastic petri dishes. Stocks of HSV-1 (Glasgow strain 17) ts^+ syn^+ (wild type) and DNA^- *ts* mutants representing eight complementation groups (1, 5, 10; *ts*B, *ts*D, *ts*H, *ts*J, *ts*K, *ts*L, *ts*S, and *ts*U) were prepared as previously described (2). Viral infectivity was assayed at 32 and 39°C, the permissive and nonpermissive temperatures, respectively. The efficiency of plating (PFU at 39°C/PFU at 32°C) was determined for all virus stocks: ts^+, 1.0; *ts*B, 4×10^{-6}; *ts*D, 6×10^{-7}; *ts*H, 8×10^{-5}; *ts*J, 1×10^{-5}; *ts*K, 1×10^{-7}; *ts*L, 5×10^{-4}; *ts*S, 1×10^{-6}; and *ts*U, 8×10^{-6}.

Temperature shift-up. Cells were washed with isotonic Tris buffer and infected with virus at 5 PFU/cell with an adsorption period of 1 h at 32°C, at which time fresh medium was added. At 11 h postinfection (p.i.), cultures were shifted up to 39°C by replacing the medium with prewarmed medium (39°C) and incubating the cultures at 39°C. Duplicate cultures were pulse-labeled with 10 μCi of [^{3}H]thymidine per plate ([^{3}H]deoxyribosylthymidine [dTHd], 55 Ci/mmol; New England Nuclear Corp.) at 32°C for 60 min at 10, 11, and 15 h p.i. and at 39°C for 30 min at 11, 11.5, 12, 13, and 14 h p.i. These pulse periods were chosen to compensate for the temperature effect on the rate of DNA synthesis. After each pulse labeling, the cells were washed twice with isotonic Tris buffer and the following solutions were added: 1.0 ml of 0.15 M NaCl and 0.01 M EDTA in 0.01 M Tris-hydrochloride (pH 8.0), 0.1 ml of 20% sodium dodecyl sulfate, and 0.1 ml of 10-mg/ml Pronase (pretreated for 30 min at 60°C in 0.2 M potassium acetate). The material from duplicate cultures was pooled and incubated overnight at 32°C.

Temperature shift-down. Cells were infected as described above. Virus was adsorbed for 0.5 h at 39°C, at which time fresh medium was added and cultures were incubated at 39°C. At 6 h p.i., duplicate cultures received 2 μCi of [^{3}H]dTHd per plate and either actinomycin D (1 μg/ml; Calbiochem), an inhibitor of DNA-dependent RNA synthesis, or cycloheximide (50 μg/ml; Aldrich Chemical Co.), an inhibitor of protein synthesis. At this time, cultures were incubated at 32°C and harvested 20 h p.i. as described above.

Quantitation of viral and cellular DNA. DNA from infected cells was analyzed by CsCl equilibrium density gradient centrifugation (2). Viral DNA banded at a buoyant density of 1.725 g/cm^3, and cellular DNA banded at a density of 1.700 g/cm^3. The values for [^{3}H]dTHd incorporation into viral and cellular DNA were obtained by integrating the ^{3}H counts per minute in the respective peaks and correcting for base-line counts per minute. If no peak of viral DNA could be detected, the ^{3}H counts per minute in the region where viral DNA would normally have appeared were integrated. These figures are given in parentheses in Tables 1 and 2 and represent the maximum possible [^{3}H]dTHd incorporation into viral DNA that would have gone undetected.

RESULTS

Viral and cellular DNA synthesis at 32°C and 39°C. Virus-infected cells were continuously incubated at 32 and 39°C and labeled with [^{3}H]dTHd to study viral and cellular DNA synthesis under permissive and nonpermissive conditions (Table 1). Cells infected with wild-type virus synthesized viral DNA at both temperatures. Mutant-infected cells synthesized various levels of viral DNA at 32°C, but no viral DNA synthesis was detected at 39°C in any mutant-infected culture. The figures that show the percent [^{3}H]dTHd incorporation into viral DNA at 39°C relative to 32°C in the mutant-infected cells represent the maximum possible incorporation that would not have been detected because of background counts per minute. These results confirm the DNA^- phenotype of the eight mutants. Wild-type virus and all of the *ts* mutants inhibited host DNA synthesis at 32°C compared to mock-infected cells (results not shown). The percent incorporation of [^{3}H]dTHd into cellular DNA in infected cells at 39°C relative to 32°C (Table 1) indicated that certain mutants, notably *ts*K, were less efficient at inhibiting cellular DNA synthesis at 39°C than they were at 32°C.

Viral DNA synthesis in wild-type and mutant-infected cells after shift-up to 39°C. Viral DNA synthesis was measured after shift-up to the nonpermissive temperature in cells infected with the wild type and eight mutants in an attempt to characterize the nature of the block in DNA synthesis. Infected cells were incubated at 32°C to allow the synthesis of viral DNA. At 11 h p.i., when viral DNA was actively being synthesized, the cultures were shifted up

TABLE 1. *[^{3}H]dTHd labeling of viral and cellular DNA at 32 and 39°C*[a]

Virus	^{3}H incorporated at 32°C (cpm)		^{3}H incorporated at 39°C (%)[b]	
	Viral DNA	Cellular DNA	Viral DNA	Cellular DNA
ts^+	341×10^3	87×10^3	58	71
*ts*B	51×10^3	181×10^3	(≤0.98)[c]	140
*ts*D	70×10^3	184×10^3	(≤0.71)[c]	159
*ts*H	195×10^3	253×10^3	(≤0.51)[c]	186
*ts*J	171×10^3	229×10^3	(≤0.58)[c]	67
*ts*K	242×10^3	184×10^3	(≤0.83)[c]	222
*ts*L	38×10^3	268×10^3	(≤0.79)[c]	86
*ts*S	46×10^3	214×10^3	(≤0.87)[c]	175
*ts*U	121×10^3	172×10^3	(≤0.83)[c]	150

[a] Virus-infected cells were incubated at 32 or 39°C, [^{3}H]dTHd was added 6 h p.i., and the cultures were harvested 20 h p.i. The DNA was analyzed by CsCl density gradient centrifugation and ^{3}H counts per minute representing viral or cellular DNA were integrated and corrected for background.

[b] (^{3}H cpm incorporated at 39°C/^{3}H cpm incorporated at 32°C) × 100.

[c] No peak of viral DNA was detected. Figure in parentheses represents maximum possible incorporation that would have gone undetected.

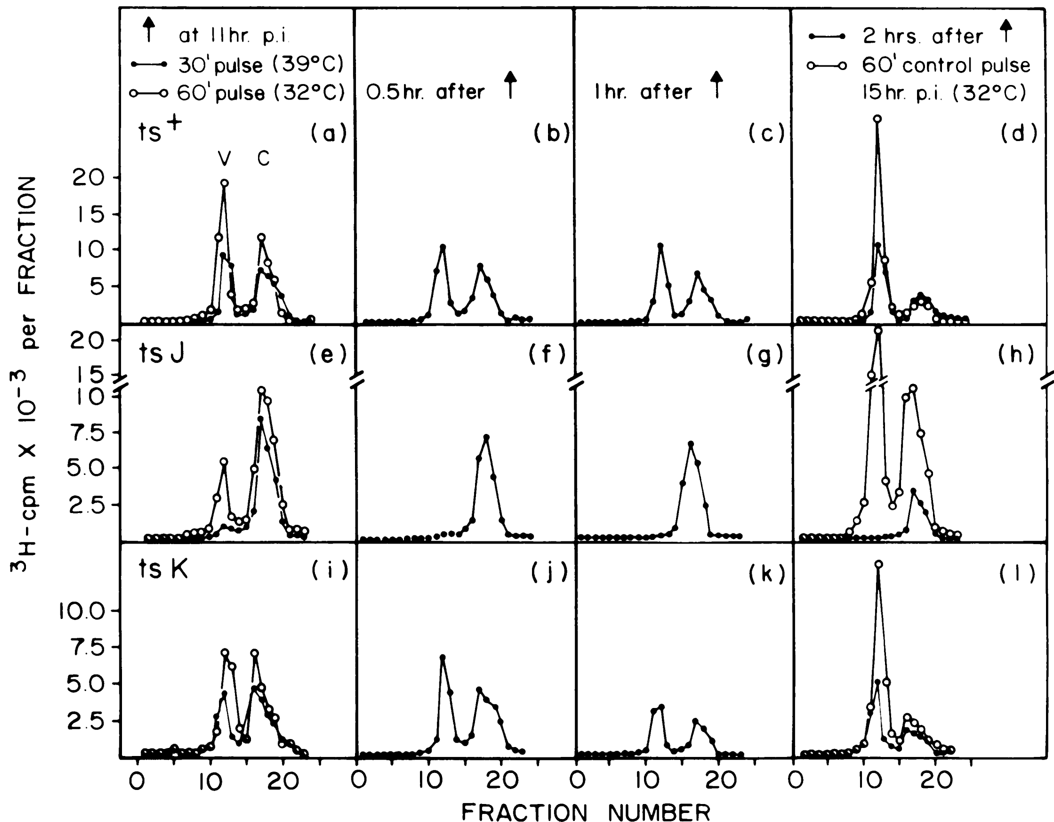

FIG. 1. *CsCl density gradient profiles of DNA from cells infected with ts+, tsJ, and tsK and pulse-labeled after shift-up. Cells infected with ts+ (a-d), tsJ (e-h), and tsK (i-l) were shifted up from 32°C to 39°C 11 h p.i. and pulse-labeled with [3H]dTHd. After each pulse, the cultures were harvested, and viral DNA (V) and cellular DNA (C) were separated in CsCl density gradients. Superimposed profiles of 30-min pulse at 39°C (●) and 60-min pulse at 32°C (○) 11 h p.i. (a, e, i); 30-min pulse at 39°C 11.5 h p.i. (b, f, j); 30-min pulse at 39°C 12 h p.i. (c, g, k); and superimposed profiles of 30-min pulse at 39°C 13 h p.i. (●) and 60-min pulse at 32°C 15 h p.i. (○) (d, h. l). Density increase is from right to left.*

to 39°C, and the rate of viral DNA synthesis was measured by a series of consecutive pulses with [^{3}H]dTHd. After each pulse, DNA was extracted and analyzed by CsCl density gradient centrifugation. The actual gradient profiles are shown in Fig. 1 for cells infected with the wild type and two mutants showing different responses after shift-up. Wild type-infected cells (Fig. 1a-d) synthesized viral DNA at all times after shift-up. Viral DNA synthesis was inhibited 0.5 h after shift-up in *ts*J-infected cells (Fig. 1e-h). Cells infected with *ts*K continued to synthesize viral DNA at all times after shift-up (Fig. 1i-l).

For each pulse labeling of cells infected with wild-type virus and each mutant, viral DNA synthesis was quantitated by integrating the ^{3}H counts per minute incorporated into viral DNA (as determined from CsCl density gradient centrifugation) and correcting for background counts per minute. The results for wild type and for *ts*J, *ts*K, and all other mutants are summarized in Fig. 2. Wild type-infected cells continued to synthesize viral DNA at a constant rate after shift-up. Cells infected with *ts*D and *ts*K most resembled wild type-infected cells. Cells infected with *ts*L, *ts*S, and *ts*U also continued to synthesize viral DNA after shift-up, but at a more variable rate. Viral DNA synthesis was inhibited after shift-up in cells infected with *ts*B, *ts*H, and *ts*J. The time at 39°C required for complete inhibition of viral DNA synthesis in cells infected with these three mutants varied from 0.5 h with *ts*J to 3 h with *ts*H. The pulse labelings at 32°C showed that each mutant-infected culture continued to synthesize viral DNA under permissive conditions.

Viral DNA synthesis in wild type- and mutant-infected cells shifted down from 39°C to 32°C in the presence and absence of inhibitors of RNA and protein synthesis.

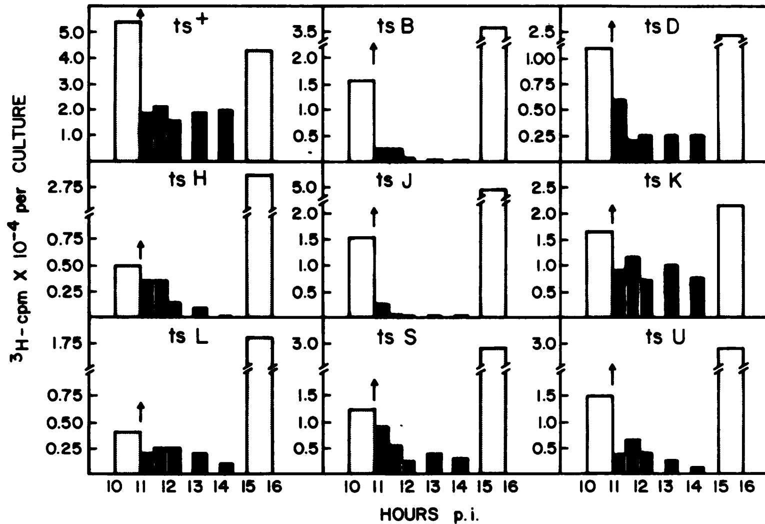

FIG. 2. *Rates of viral DNA synthesis in cells infected with ts+ and eight ts mutants and pulse-labeled before and after shift-up. Virus-infected cells were shifted up from 32°C to 39°C 11 h p.i. and pulse-labeled with [3H]dTHd: 30-min pulses at 39°C at 11, 11.5, 12, 13, and 14 h p.i. (solid bars) and 60-min pulses at 32°C at 10 and 15 h p.i. (dotted bars). After each pulse, DNA was extracted and analyzed in CsCl density gradients (as detailed in Fig. 1). The ^{3}H counts per minute representing viral DNA were integrated and corrected for background counts per minute. The time of temperature shift-up is indicated by the arrow.*

To determine whether the de novo synthesis of RNA and protein was required for the synthesis of viral DNA, cells infected with the wild type and the eight mutants were shifted down to the permissive temperature in the presence and absence of inhibitors of RNA and protein synthesis. Three sets of infected cultures were shifted down to 32°C after 6 h at 39°C: (i) one set received [^{3}H]dTHd and 1 μg of actinomycin D per ml; (ii) another received [^{3}H]dTHd and 50 μg of cycloheximide per ml; and (iii) the third set received only [^{3}H]dTHd. At 20 h p.i., DNA was extracted and analyzed by CsCl density gradient centrifugation (Table 2). In the presence of actinomycin D and cycloheximide, wild type-infected cells synthesized 18 and 25%, respectively, of the viral DNA synthesized by the control culture which received no inhibitor. Cells infected with *ts*B, *ts*H, *ts*L, *ts*S, and *ts*U synthesized viral DNA in the presence of either inhibitor, and *ts*J-infected cells synthesized viral DNA in the presence of actinomycin D but not in the presence of cycloheximide. Cells infected with *ts*D and *ts*K did not synthesize viral DNA in the presence of either inhibitor.

In a temperature shift-down experiment, the situation with wild type-infected cells is necessarily different from that for cells infected with DNA$^-$ mutants. The incorporation of [^{3}H]dTHd after shift-down will measure continued DNA synthesis in wild type-infected cells while the mutant-infected cells have to begin viral DNA synthesis. The degree of recovery (the quantity of viral DNA synthesized after shift-down) may vary from one mutant to another and will de-

TABLE 2. *[^{3}H]dTHd labeling of viral DNA after shift-down from 39°C to 32°C in the presence and absence of actinomycin D and cycloheximide*[a]

Virus	^{3}H incorporated without inhibitor (cpm)	^{3}H incorporated in the presence of inhibitor (%)[b]	
		Actinomycin D	Cycloheximide
ts+	290 × 10^3	18	25
*ts*B	79 × 10^3	2	15
*ts*D	27 × 10^3	(≤0.74)[c]	(≤0.74)[c]
*ts*H	157 × 10^3	5	2
*ts*J	157 × 10^3	3	(≤0.64)[c]
*ts*K	16 × 10^3	(≤0.62)[c]	(≤0.62)[c]
*ts*L	68 × 10^3	15	6
*ts*S	87 × 10^3	7	2
*ts*U	98 × 10^3	14	11

[a] Virus-infected cells were shifted down from 39°C to 32°C at 6 h p.i., and [^{3}H]dTHd and inhibitors (1 μg of actinomycin D per ml or 50 μg of cycloheximide per ml) were added. At 20 h p.i., the cultures were harvested and viral DNA was quantitated as described in Table 1.

[b] (^{3}H cpm incorporated in the presence of inhibitor/^{3}H cpm incorporated in the absence of inhibitor) × 100.

[c] No peak of viral DNA was detected. Figure in parentheses represents maximum possible incorporation that would have gone undetected.

pend on the stability of the input mutant genomes under nonreplicating conditions.

DISCUSSION

The eight *ts* DNA$^-$ mutants of HSV-1 can be divided into three categories based on the results of the temperature-shift studies presented above. (i) Cells infected with *ts*B, *ts*H, and *ts*J were inhibited in the synthesis of viral DNA after a shift-up to the nonpermissive temperature. It is, therefore, likely that the B, H, and J proteins are continuously required for the synthesis of viral DNA. The length of time at 39°C required to inactivate the gene product ranged from 0.5 h with *ts*J to 3 h with *ts*H. These inactivation times may reflect that *ts*J was defective in the elongation of viral DNA, since an elongation-defective mutant would cease DNA synthesis soon after shift-up, and that *ts*B and *ts*H were defective in the initiation of viral DNA synthesis, since initiation-defective mutants would complete rounds of replication begun at the permissive temperature. Mutant *ts*H codes for the synthesis of a thermolabile viral DNA polymerase (I. Crombie, Ph.D. thesis, University of Glasgow, Glasgow, Scotland, 1975) and should behave as an elongation-defective mutant unless the viral polymerase was stabilized by its association with DNA and could function to complete rounds of replication at 39°C. The polymerase would not exhibit its temperature sensitivity until it dissociated from DNA, thereby giving the phenotype of a defect in initiation.

Cells infected with *ts*B and *ts*H synthesized viral DNA after shift-down in the absence of de novo RNA and protein synthesis. It is likely that the defective B and H proteins synthesized at 39°C could assume a functional conformation after shift-down to 32°C and then could act to synthesize viral DNA even in the presence of actinomycin D or cycloheximide. Cells infected with *ts*J synthesized viral DNA after shift-down in the presence of actinomycin D but not in the presence of cycloheximide. De novo protein synthesis after shift-down would be required for viral DNA synthesis if the J protein synthesized at 39°C could not assume a functional conformation at 32°C, if the J protein synthesized at 39°C was degraded prior to shift-down, or if the translation of RNA coding for the J protein was temperature sensitive.

Schaffer et al. (9) reported that mutants in three of four DNA$^-$ complementation groups synthesized little or no viral DNA after shift-up to the nonpermissive temperature. In this communication we report that mutants in three of eight DNA$^-$ complementation groups were inhibited in the synthesis of viral DNA after shift-up. It is clear that at least three viral proteins are continuously required for the synthesis of viral DNA and that one of the proteins is the DNA polymerase.

(ii) Because the block to viral DNA synthesis at 39°C in cells infected with *ts*D and *ts*K can be bypassed by prior incubation at 32°C and since viral DNA synthesis after shift-down required de novo RNA and protein synthesis, *ts*D and *ts*K are likely to be defective in the expression of regulatory genes whose products are required early in the replication cycle. Cells infected with HSV and incubated in the presence of cycloheximide accumulate certain viral polypeptides when the inhibitor is removed. Rakusanova et al. (6) have called them immediate early polypeptides, and Honess and Roizman (3) have called them α-polypeptides. Marsden et al. (5) reported that *ts*D was deficient in the turn-off of α-polypeptide synthesis. Mutant *ts*K has been shown to be extremely limited in the extent of viral transcription, and *ts*D and *ts*K have been shown to map very closely together on the viral genome (N. Wilkie, personal communication). Schaffer et al. (9) reported that cells infected with their *ts*B continued to synthesize viral DNA after shift-up to the nonpermissive temperature, a result similar to our observations with *ts*D. Both mutants appear to be defective in the same gene since they fail to complement each other (P. Schaffer, personal communication).

(iii) Mutants *ts*L, *ts*S, and *ts*U are difficult to classify on the basis of the results presented above. Viral DNA was synthesized after shift-down in the absence of RNA and protein synthesis, indicating that these mutants were not defective in early regulatory functions. Cells infected with these mutants continued to synthesize viral DNA after shift-up, signifying that the L, S, and U proteins were not continuously required for the synthesis of viral DNA. Since the shift-down was performed at 11 h p.i., we cannot exclude the possibility that the L, S, and U proteins function exclusively during the early stages of DNA replication and are not required during the later stages when viral DNA may be synthesized by a potentially different mechanism. A more likely explanation is that these mutants are "leaky," even though these experiments were performed at 39°C instead of 38.5°C, the nonpermissive temperature used for mutant isolation. The isolation and characterization of additional, nonleaky mutants in these three complementation groups would help to clarify the function of these viral gene products.

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