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DNA-Negative Temperature-Sensitive Mutants of Herpes Simplex Virus Type 1: Patterns of Viral DNA Synthesis After Temperature Shift-Up

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Temperature-sensitive mutants of herpes simplex virus type 1 belonging to four DNA$^-$ complementation groups exhibited two distinct patterns of viral DNA synthesis after shift-up to the nonpermissive temperature. In cultures infected with mutants belonging to complementation groups A, C, and D, little or no viral DNA was synthesized after shift-up. In cultures infected with a mutant in complementation group B, nearly normal amounts of viral DNA were synthesized after shift-up.

Evidence implicating a large number of herpes simplex virus (HSV) genes in the control of viral DNA synthesis has come from studies with temperature-sensitive (ts) mutants of HSV in this and other laboratories (3, 5, 6, 9). We have demonstrated that at least four genes control the synthesis of HSV type 1 (HSV-1) DNA (1, 8). However, little is currently known about the specific roles these genes play in viral DNA synthesis. Previous studies have shown that no viral DNA synthesis occurs in cells infected with ts mutants in complementation groups A, B, C, or D and incubated continuously at the nonpermissive temperature (9). The purpose of the present study was to examine viral DNA synthesis by HSV-1 ts mutants representing the four DNA$^-$ complementation groups in temperature shift-up experiments in an effort to answer the following questions: (i) Does viral DNA synthesis occur at the nonpermissive temperature when cells are preincubated at the permissive temperature? (ii) If so, what is the time of preincubation at the permissive temperature required to pass the temperature-sensitive step? (iii) If the temperature-sensitive step can be passed, are normal levels of viral DNA synthesized? The results of these studies demonstrate that mutants in the four complementation groups exhibit two distinct patterns of viral DNA synthesis after shift-up to the nonpermissive temperature.

Human embryonic lung cells were used in this study. The methods used for their growth and maintenance have been described previously (9). Three to six million human embryonic lung cells in 8-oz (about 240-ml) prescription bottles were infected at a multiplicity of 10 PFU/cell with the wild-type (WT) virus and with five DNA$^-$ ts mutants of HSV-1 (tsA15 and tsA16; tsB2; tsC4 and tsD9) representing four complementation groups (9). Infected cells were incubated at 37 C for 1 h, 10 ml of maintenance medium was added to each culture, and all bottles were incubated at the permissive temperature (34 C) (Fig. 1). At 2, 3, 4, 5, 6, and 8 h postinfection (p.i.), one culture was shifted to a water bath maintained at the nonpermissive temperature (39 ± 0.1 C). These times were selected since viral DNA synthesis has been shown to be maximal for both the WT virus and the mutants at 34 C in human embryonic lung cells from 2 to 8 h p.i. (unpublished observations). At the time of shift-up, cultures were labeled with 10 μCi of [^{3}H]thymidine per ml ([^{3}H]TdR, 52 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.). Parallel cultures maintained at 34 C were labeled in a similar manner. All cultures were harvested 24 h p.i. and processed for DNA determination as described previously (1) with the following modifications. A volume of each infected-cell lysate containing approximately 1.5×10^5 trichloroacetic acid-precipitable counts was made up to a total volume of 4.5 ml in TNE buffer (0.01 M Tris-chloride, pH 7.4, containing 0.01 M NaCl and 0.001 M EDTA), and the density was adjusted to 1.708 g/cm^3 with CsCl. Each 4.5-ml sample was placed in a Beckman polyallomer tube and overlaid with 0.2 ml of mineral oil. Suspensions were centrifuged in an SW56 rotor at 208,216 × *g* for 48 h at 25 C. For each gradient, 45 0.1-ml fractions were collected (ISCO density gradient fractionator, model 640; Instrumentation Specialties Co., Lincoln, Neb.), refractive in-

dexes of selected fractions were determined, and 50 μl of each fraction was placed on a Whatman GF/A filter paper disk. Disks were dried, processed, and counted (1). To make results obtained with different viruses comparable, counts in each fraction were adjusted to reflect a sample containing 10^6 cells in each gradient.

Patterns of incorporation of [^{3}H]TdR into DNA by WT virus and ts mutant-infected cells are shown in Fig. 2 through 5. Two species of DNA were observed in lysates of [^{3}H]TdR-labeled, virus-infected cells; one species that banded at a buoyant density of 1.700 g/cm^3 corresponded to cellular DNA and one, which banded at 1.725 g/cm^3, corresponded to viral DNA (Fig. 2).

WT virus-infected cultures labeled at increasingly later times p.i. exhibited a successive increase in the ratio of labeled viral to cellular

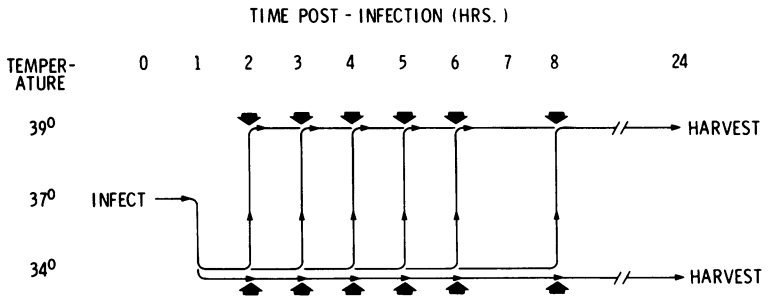

FIG. 1. *Diagram of temperature shift-up experiments. Virus was adsorbed to monolayers for 1 h at 37 C when all cultures were shifted to 34 C. At the indicated times, cultures were shifted to 39 C and labeled (⬇) with [^{3}H]thymidine. Control cultures were maintained at 34 C and labeled (⬆) at the same times as shifted cultures. All cultures were harvested 24 h postinfection.*

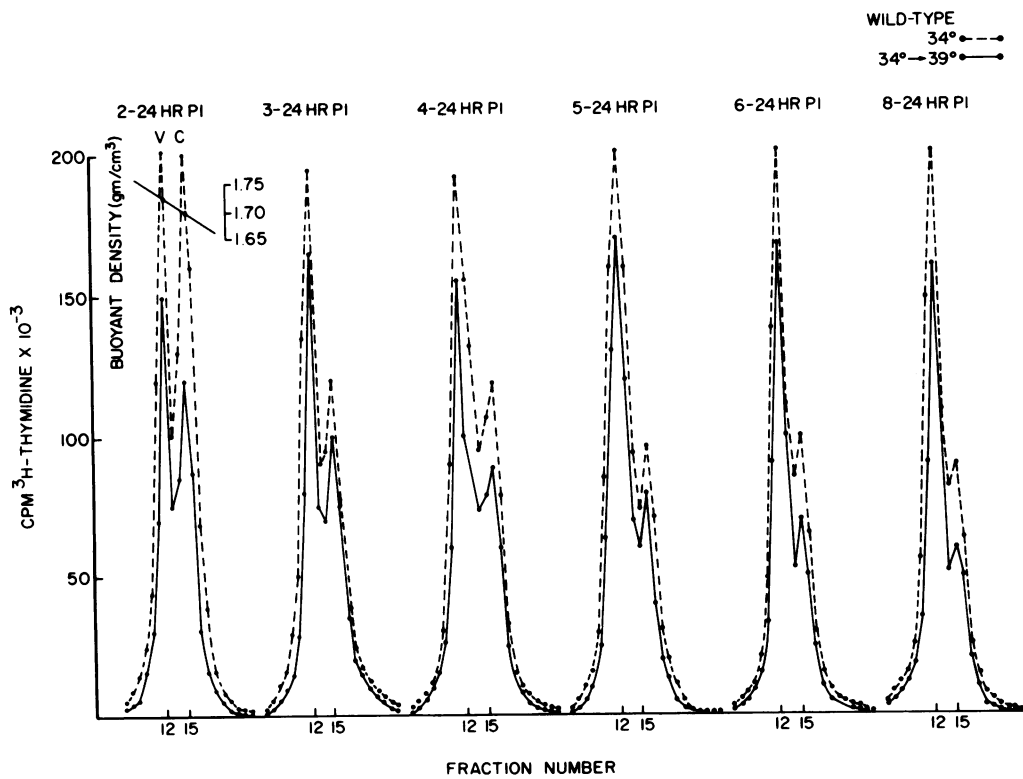

FIG. 2. *Profiles of [^{3}H]thymidine-labeled viral (V) and cellular (C) DNA obtained by cesium chloride density gradient centrifugation of lysates of wild-type virus-infected cultures after shift-up to 39 C at 2 to 8 h postinfection and of unshifted cultures labeled similarly.*

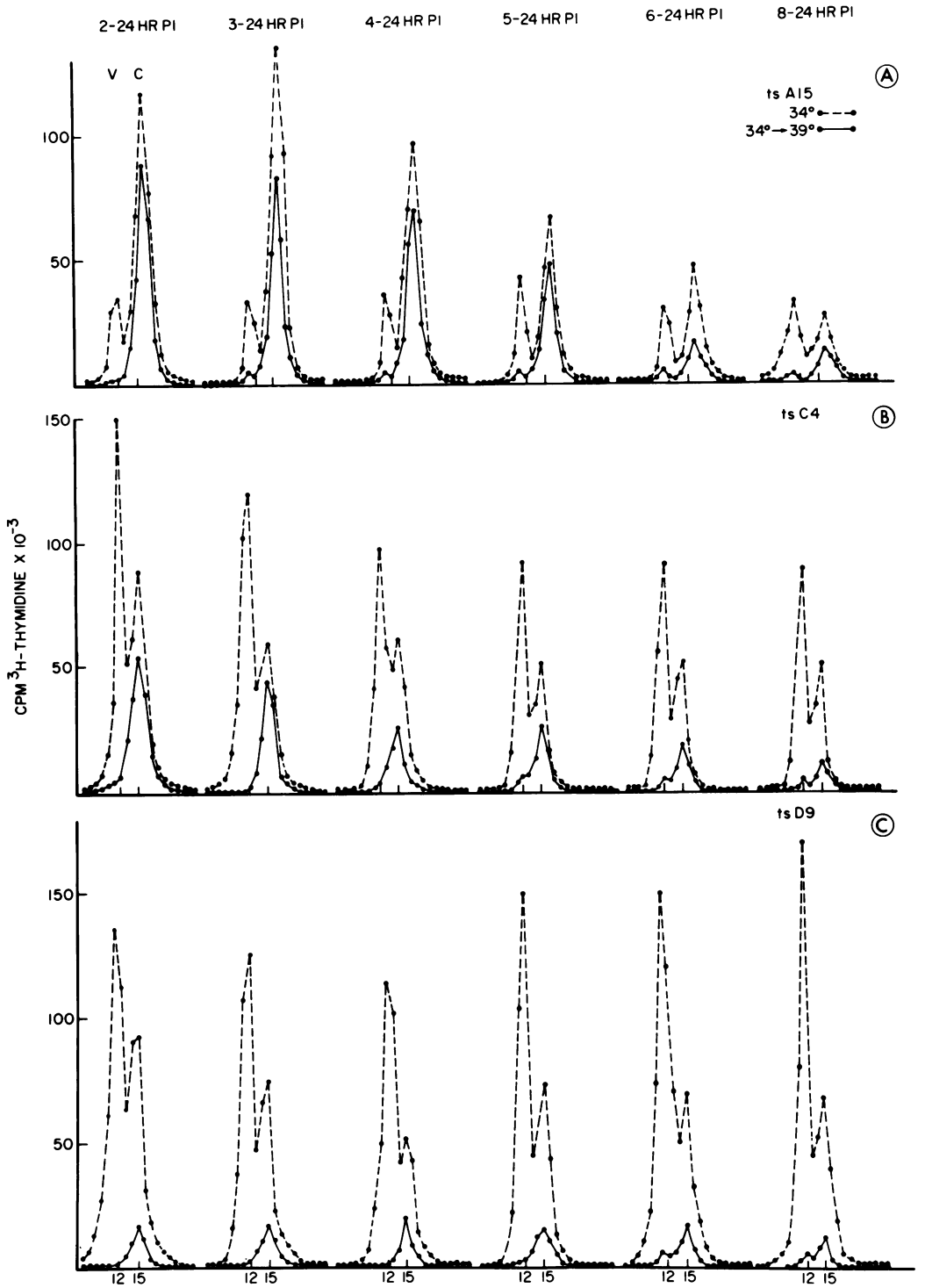

FIG. 3. *Profiles of [^{3}H]thymidine-labeled viral (V) and cellular (C) DNA obtained by cesium chloride density gradient centrifugation of lysates of tsA15- (A), tsC4- (B), and tsD9- (C) infected cultures after shift-up to 39 C at 2 to 8 h postinfection and of unshifted cultures labeled similarly.*

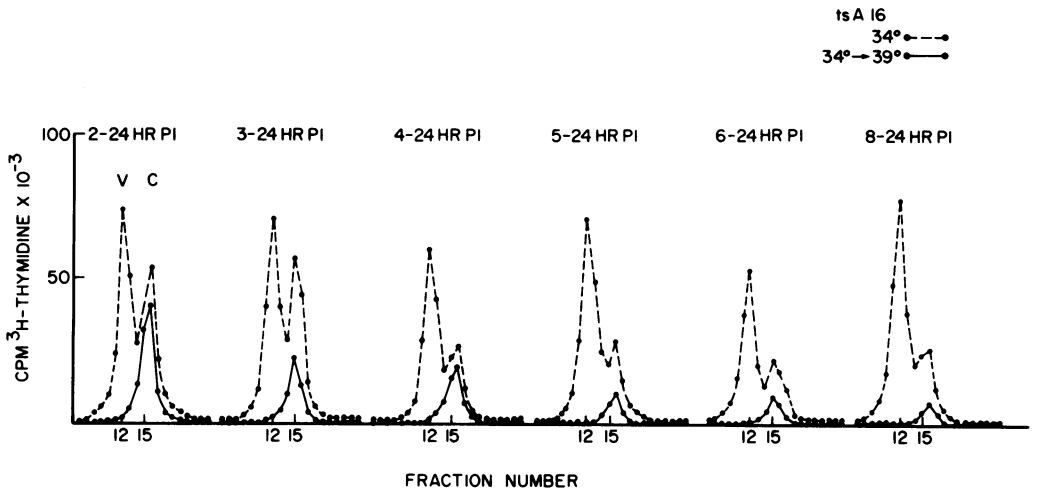

FIG. 4. *Profiles of [³H]thymidine-labeled viral (V) and cellular (C) DNA obtained by cesium chloride density gradient centrifugation of lysates of tsA16-infected cultures after shift-up to 39 C at 2 to 8 h postinfection and of unshifted cultures labeled similarly.*

DNA (Fig. 2). Although slightly less labeled DNA was observed in shifted cultures, patterns of incorporation of [³H]TdR into both species of DNA were similar in cultures maintained at 34 C or in those shifted from 34 to 39 C at all times tested.

Two distinct patterns of incorporation of [³H]TdR into viral and cellular DNA were observed in DNA⁻ mutant-infected cultures after shift-up.

(i) Cultures infected with tsA15, tsA16, tsC4, and tsD9 synthesized little or no viral DNA when shifted from 34 to 39 C, and cellular DNA synthesis was more rapidly shut off at 39 C than at 34 C (Fig. 3 and 4). tsA15, tsC4, and tsD9 synthesized small amounts of viral DNA at the nonpermissive temperature after shift-up (Fig. 3). The time of preincubation at 34 C required before viral DNA synthesis was detected at 39 C varied with each of the three mutants: 3 h for tsA15, 5 h for tsC4, and 6 h for tsD9. Despite the variation in the time of first appearance of viral DNA in shifted cultures, the amount of DNA synthesized was approximately the same for each of the three mutants. These observations suggest that the gene products required for viral DNA synthesis were active for only a short time after shift to 39 C, resulting in the production of a limited amount of DNA.

No incorporation of [³H]TdR into viral DNA was detected in cultures infected with tsA16 after shift-up at all times tested (Fig. 4), indicating that the gene product required for viral DNA synthesis was immediately and totally nonfunctional in cultures shifted to 39 C. The reason for the observed differences in the patterns of viral DNA synthesis after temperature shift-up by the two mutants in complementation group A is not known. It is possible, however, that the ts mutation in tsA16 had a greater effect on the ability of the defective gene product to function at 39 C than did the mutation in tsA15; i.e., the tsA16 mutation was less leaky than the tsA15 mutation. Thus, since little or no viral DNA was synthesized at 39 C by group A, C, or D mutants after preincubation at 34 C for 2 to 8 h p.i., the presence of at least three distinct gene products is required continuously during this time for viral DNA synthesis to occur.

Cellular DNA synthesis was rapidly depressed after infection with all four mutants (see 2- to 24-h gradients, Fig. 3 and 4). Furthermore, decreasing amounts of cellular DNA were labeled in successive cultures, probably due to the progressive shut-off of cellular DNA synthesis with increasing time p.i. and to the shorter labeling period.

(ii) In contrast to the four mutants described above, the amount of labeled viral DNA synthesized at the nonpermissive temperature in cultures infected with tsB2 increased significantly as a function of time of shift p.i., despite the decreased labeling time (Fig. 5). Indeed, the increase was so extensive in cultures shifted to 39 C at 8 h p.i. that the amount of viral DNA synthesized was nearly identical to that in unshifted cultures. Furthermore, after only 2 h preincubation at lower temperature (1 h at 37 C and 1 h at 34 C) and at all times of shift

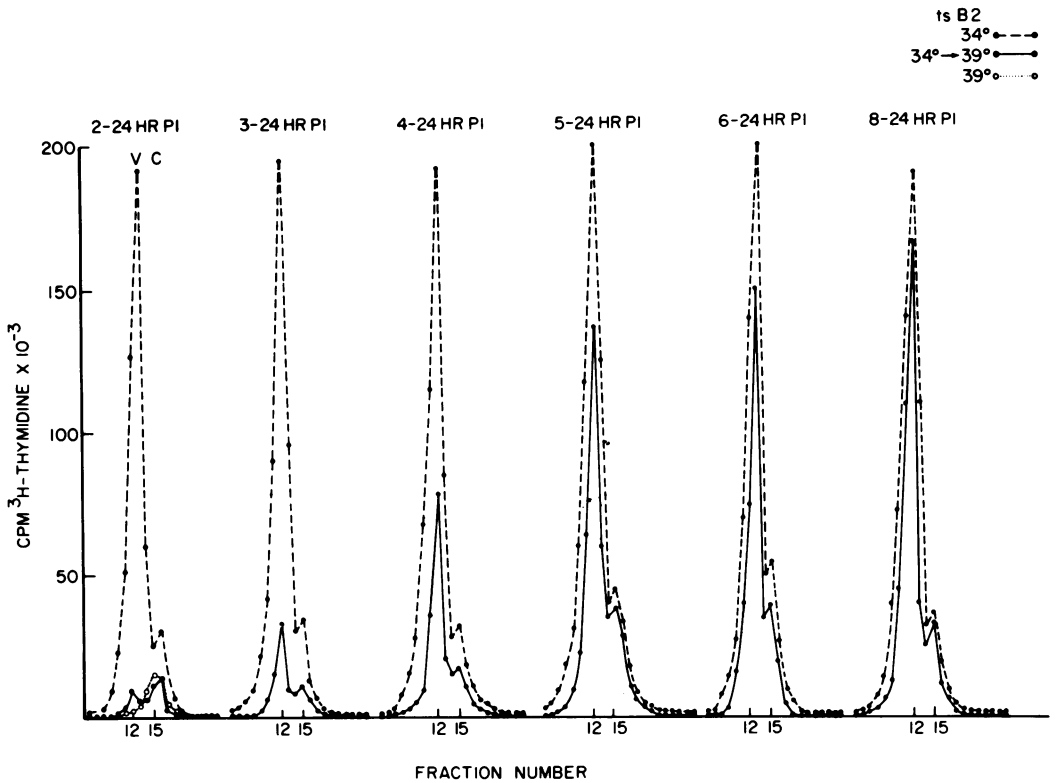

FIG. 5. *Profiles of [³H]thymidine-labeled viral (V) and cellular (C) DNA obtained by cesium chloride density gradient centrifugation of lysates of tsB2-infected cultures after shift-up to 39 C at 2 to 8 h postinfection and unshifted cultures labeled similarly. The profile of a control, tsB2-infected culture maintained at 39 C from 1 to 24 h and labeled from 2 to 24 h postinfection is also shown.*

thereafter, viral DNA was synthesized at 39 C. On the other hand, in cultures shifted to 39 C after 1 h of incubation at 37 C and labeled from 2 to 24 h p.i., no peak of viral DNA was evident (Fig. 5, first gradient, open circles). Results with tsB2 thus indicate that after the synthesis of a functional gene product (before 2 h p.i.), viral DNA synthesis occurs normally at the nonpermissive temperature.

Although cellular DNA synthesis was rapidly and maximally depressed in tsB2-infected cells maintained at 39 C from 2 to 24 h p.i., the amount of labeled cellular DNA also increased as a function of time of shift p.i., despite the decreased labeling period. This observation indicates that the synthesis of cellular as well as viral DNA was dependent upon the presence of functional tsB2 gene product early after infection. Whether the defects of the mutants used in this study affect functions involved directly in viral DNA synthesis or whether they involve events before viral DNA synthesis is currently being investigated. With regard to the ability of these mutants to induce the synthesis of virus-specific DNA polymerase (DP), mutants in group A induced levels of DP similar to that of the WT virus, mutants in group B were deficient in DP activity at 39 C but not at 34 C, and mutants in groups C and D induced the synthesis of a temperature-sensitive DP as demonstrated by in vivo studies (1). The patterns of viral DNA synthesis after temperature shift-up exhibited by mutants in group A thus do not result from defects in viral DP. The same can probably be said for mutants in group B since the ts defect in these mutants is early (before 2 h p.i.) and transient whereas viral DP activity is maximal later (2 to 8 h p.i.) in infection and is required continuously during this period. Mutants in groups C and D, on the other hand, exhibited patterns of viral DNA synthesis after shift-up that are compatible with their ts DP phenotypes. As previously suggested, the limited synthesis of viral DNA after shift-up induced by these mutants may have occurred before the ts DP was completely inactivated at 39 C.

With regard to the association between the

synthesis of viral DNA and virus-specific proteins, DNA$^-$ ts mutants exhibit more pronounced defects in polypeptide synthesis than do most DNA$^+$ mutants (R. J. Courtney, P. A. Schaffer, and K. L. Powell, manuscript in preparation). Of the group A mutants, tsA15 exhibited a polypeptide profile typical of most DNA$^-$ mutants, i.e., nearly normal or increased amounts of early polypeptides and reduced amounts of late polypeptides. tsA16, on the other hand, was more severely limited in polypeptide synthesis since reduced amounts of all (early and late) virus-induced polypeptides occurred at the nonpermissive temperature. Whether the inability of tsA16 to induce viral DNA synthesis after shift-up reflects its limited ability to induce viral polypeptide synthesis is presently not known.

Regarding mutants in group C, we have previously reported that tsC4 was defective in the synthesis of the major capsid polypeptide, VP154, at the nonpermissive temperature (2). In temperature shift-up studies, the expression of the defect relating to VP154 only occurred before 4 h p.i. These data suggested that the defect is early in the replicative cycle and that the gene product essential for VP154 synthesis retains its activity after shift to 39 C. However, this was not the case in the shift-up studies of tsC4 and viral DNA synthesis, as described above. These data would thus suggest no direct relationship between the synthesis of VP154 and viral DNA synthesis (7, 8). The polypeptide profile of the group D mutant tsD9 is similar to that described above for tsA15.

Extensive studies of polypeptide synthesis by tsB2 have demonstrated that this mutant induces markedly elevated amounts of a large-molecular-weight, nonstructural polypeptide, VP175 (4). This polypeptide belongs to the alpha class of early polypeptides of HSV-1 described by Honess and Roizman (7) and is similar to the polypeptide that they designated ICP4. Temperature shift-up studies of tsB2 indicated that the block responsible for the elevated synthesis of VP175 occurred before 4 h p.i. (4), supporting the conclusion drawn in the present study (i.e., that the block in viral DNA synthesis occurred before 2 h p.i.). Although no definitive correlations can yet be drawn between the synthesis of specific HSV polypeptides and the control of viral DNA synthesis, additional studies of these processes using DNA$^-$ ts mutants should help to define these correlations.

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