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Abnormal Properties of an Immediate Early Polypeptide in Cells Infected with the Herpes Simplex Virus Type 1 Mutant *tsK*

C. M. PRESTON

Medical Research Council Virology Unit, Glasgow G11 5JR, Scotland

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Previous studies (R. J. Watson and J. B. Clements, Virology **91**:364-379, 1978; C. M. Preston, J. Virol. **29**:275-284, 1979) have shown that the herpes simplex virus type 1 (HSV-1) mutant *tsK* has a temperature-sensitive lesion in an immediate early polypeptide whose function is to induce synthesis of new viral transcripts, including mRNA, for pyrimidine deoxyribonucleoside kinase. The studies presented here examine the properties of immediate early polypeptides in wild-type HSV-1- and *tsK*-infected cells at 31 and 38.5°C. The overall pattern of immediate early protein synthesis was similar in wild-type HSV-1- and *tsK*-infected cells when radiolabeled with [^{35}S]methionine or ^{14}C-amino acid mixture. Further investigation, however, revealed two aberrant properties of the polypeptide V_{mw} 175 in *tsK*-infected cells at 38.5°C. Upon cell fractionation, large amounts of this polypeptide were recovered in the cytoplasmic fraction, in contrast to *tsK*-infected cells at 31°C or wild-type HSV-1-infected cells at either temperature. Furthermore, at 38.5°C *tsK*-induced V_{mw} 175 was not processed normally to forms of lower electrophoretic mobility. Both of these defects were reversible upon downshift of *tsK*-infected cells, even in the absence of further protein synthesis, but were not observed in cells infected with a revertant of *tsK*. Coinfection of *tsK*-infected cells with wild-type HSV-1 did not alleviate these lesions, suggesting that they resulted from an abnormal V_{mw} 175 polypeptide rather than from a defective processing enzyme. Temperature upshift of *tsK*-infected cells caused reversion of V_{mw} 175 to the mutant form. The progression to synthesis of late polypeptides was also arrested; therefore, a functional lesion was also reversible upon temperature changes between 31 and 38.5°C during the early stages of infection. The identification of a polypeptide with abnormal properties in *tsK*-infected cells and the demonstration that these properties, and the functional lesion, are reversible may provide an important system for investigation of HSV-1 transcriptional control.

Infection of cultured mammalian cells with herpes simplex virus (HSV) results in a controlled appearance of virus-induced mRNA and proteins (1, 7, 13, 17, 23). An early event is transcription of a limited portion of the input genome to give products known as immediate early (IE) RNA (1, 20, 23), or α-RNA (10). Synthesis of IE RNA is amplified if protein synthesis is arrested by the addition of cycloheximide at the time of infection, and immediately upon removal of the inhibitor IE RNA is translated to give IE polypeptides (7). These polypeptides probably have important functions in the early stages of the viral replication cycle.

The transition from synthesis of IE polypeptides to later products requires the synthesis of further RNA species; therefore, a role in transcriptional control has been postulated for IE polypeptides (7). Further evidence for this view has been obtained by studying the induction of the mRNA for the HSV-specified enzyme pyrimidine deoxyribonucleoside kinase (dPyK) (11, 18). It was shown that cells containing only IE virus-induced polypeptides were capable of synthesizing functionally active dPyK mRNA (assayed by in vitro translation or in vivo) even in the absence of further protein synthesis. Moreover, a temperature-sensitive (*ts*) mutant, *tsK*, was found to be defective in this property at the nonpermissive temperature. One of the conclusions from this work was that *tsK* possesses a *ts* lesion in an IE polypeptide whose function at the transcriptional level is necessary for the production of dPyK mRNA and other mRNA's.

The results presented here show *ts* aberrant

properties of an IE polypeptide produced in *tsK*-infected cells and furthermore suggest that the polypeptide is required in a functional state for the synthesis of many viral mRNA's.

MATERIALS AND METHODS

Cells and virus. BHK clone 13 cells (12) were used throughout. Wild-type (wt) HSV type 1 (HSV-1) was strain 17, and *tsK* was a mutant originally isolated from strain 17 by I. K. Crombie. A spontaneous revertant of *tsK* was isolated at 38.5°C by D. Dargan and plaque purified three times at 31°C. The revertant showed wt growth and polypeptide production at 31 and 38.5°C (D. Dargan and J. H. Subak-Sharpe, personal communication).

Culture and radiolabeling of cells. Cell cultures were radiolabeled by incubation in phosphate-buffered saline containing 100 μCi of [^{35}S]methionine per ml or 10 μCi of ^{14}C-amino acid mixture per ml (The Radiochemical Centre, Amersham, England). When used, cycloheximide was added at 200 μg/ml. This inhibitor was removed from cells by washing three times in culture medium at a temperature of 37 to 40°C (18). Temperature upshift or downshift was achieved by rapidly replacing the culture medium with fresh medium equilibrated to the required temperature.

Cell fractionation. Cell monolayers (approximately 10^6 cells) were washed with ice-cold phosphate-buffered saline, scraped into phosphate-buffered saline, and pelleted by centrifugation. The pellet was suspended in 200 μl of lysis buffer (10 mM Tris-hydrochloride, pH 7.5; 2 mM $MgCl_2$; 10 mM NaCl; 5 mM β-mercaptoethanol; 0.5% [vol/vol] Nonidet P-40). The cells were mixed vigorously on a Whirlmixer three times over a period of 5 min. The lysate was centrifuged at 2,000 × *g* for 2 min, and the supernatant (cytoplasmic fraction) was stored. The nuclear pellet was suspended in 1 ml of sucrose buffer (10 mM Tris, pH 7.5; 2 mM $MgCl_2$; 10 mM NaCl; 0.32 M sucrose) and centrifuged at 2,000 × *g* for 4 min. The pellet from this stage (nuclei) was denatured in 150 μl of electrophoresis sample buffer (13). A 100-μl sample of a threefold-concentrated sample buffer was added to the cytoplasmic fraction. Samples were heated at 100°C for 3 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In all cases, equivalent proportions of samples were loaded, without any attempt to equalize radioactivity.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Two gel systems were used for analysis of [^{35}S]methionine-labeled polypeptides. Since the detection of different forms of IE polypeptides was desired, cell fractions were analyzed on 9 to 18% or 6 to 15% gradient polyacrylamide gels containing 2.6% diallyltartardiamide (DATD) as cross-linker (6, 15). Electrophoresis was as described previously (6) except that the stacking gel was 5% instead of 3% polyacrylamide. One experiment (shown in Fig. 9 and 10) was designed to analyze late polypeptides. In this case, gradients of 6 to 15% polyacrylamide containing 1.5% methylenebisacrylamide (MBA) were used exactly as described by Marsden et al. (13) to avoid problems in correlating known late polypeptides of HSV-1 strain 17 with those seen by others in DATD-cross-linked gels.

Gels cross-linked with DATD were dried immediately after running, whereas those cross-linked with MBA were stained and destained before drying (13). Dried gels were subjected to autoradiography.

Quantitation of radioactivity in polypeptide bands. Autoradiograms were scanned with a Joyce-Loebl microdensitometer under conditions in which darkening of the film was proportional to the radioactivity in the source (13). The traced peaks were then cut out and weighed.

For the meaningful expression of results, the radioactivity in IE polypeptides was compared with that in the cellular actin peak. Analysis of numerous autoradiograms showed that the distribution of actin after labeling with [^{35}S]methionine and incubation in the presence of cycloheximide did not vary significantly between *tsK* and wt HSV-1-infected cells or between 31 and 38.5°C. A mean value of 40% recovered in the nuclear fraction, with a range of 31 to 47%, was obtained. Overall relative incorporation of [^{35}S]methionine into IE polypeptides varied no more than twofold between experiments and was constant for any given virus preparation-cell culture combination. The variation was probably due to differences in the exact multiplicity of infection and particle/PFU ratios of virus stocks.

An alternative method of expressing results was to calculate the percentage of a radioactive polypeptide in the nuclear fraction. In this case, values were normalized to a standard of 40% for actin (see above), but this adjustment made no substantial difference in the results obtained.

RESULTS

IE polypeptides induced by wt HSV-1 and *tsK*. Previous studies indicated that the mutant *tsK* possessed a *ts* lesion in an IE polypeptide and that the defect could be functionally reversed upon temperature downshift to 31°C (18). Therefore, investigations were initially directed toward detecting any properties of IE polypeptides which displayed similar behavior.

A comparison of IE polypeptides, labeled with [^{35}S]methionine and ^{14}C-amino acid mixture, induced by wt HSV-1 and *tsK* is shown in Fig. 1. Six major species were evident; from their apparent molecular weights it is straightforward to identify four of these as V_{mw} 175, V_{mw} 136, V_{mw} 110, and V_{mw} 63, described by Preston et al. (19), and ICP4, ICP6, ICP0, and ICP27, as designated by Pereira et al. (16), using the DATD-cross-linked gel system. The IE polypeptide V_{mw} 68 has also been described by Preston et al. (19). Polypeptide V_{mw} 12 has been detected in MBA-cross-linked gels (D. McDonald, M. Suh, and H. Marsden, manuscript in preparation), and a species of equivalent molecular weight is present in the products of in vitro translation of IE mRNA (25).

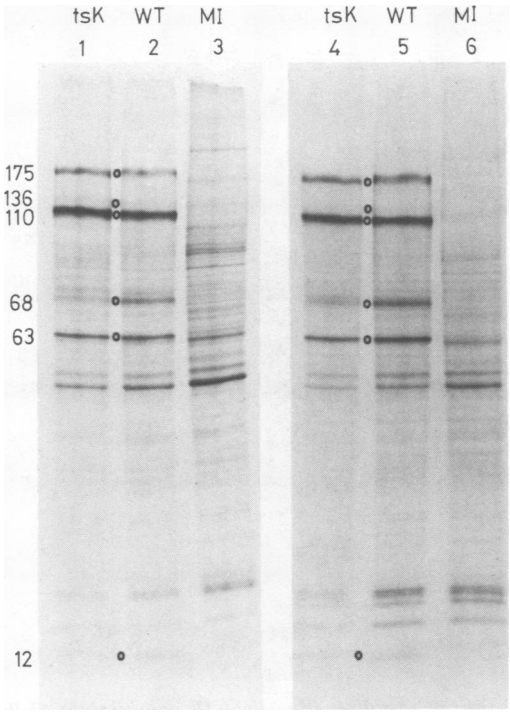

FIG. 1. *Major IE polypeptides induced by wt HSV-1 and tsK. BHK cells were infected at 38.5°C in the presence of cycloheximide and incubated for 5 h. Cycloheximide was then removed by washing, and cells were pulse-labeled with [^{35}S]methionine (tracks 1 to 3) or ^{14}C-amino acid mixture (tracks 4 to 6) for 25 min. Labeling medium was then replaced with medium containing cycloheximide, and after 1 h total cell polypeptides were harvested and analyzed on 9 to 18% gradient DATD-cross-linked gels. Tracks 1 and 4 show tsK-infected cell polypeptides, tracks 2 and 5 show wt HSV-1-infected cell polypeptides, and tracks 3 and 6 show mock-infected (MI) cell polypeptides. IE polypeptides* (○) *are labeled.*

The IE polypeptide profiles of wt HSV-1 and *tsK* differ only in the electrophoretic mobility of V_{mw} 175, as discussed below, and no additional species were revealed when cells were labeled with ^{14}C-amino acid mixture instead of [^{35}S]methionine. It should be noted that polypeptides induced by *tsK* at the nonpermissive temperature in the absence of cycloheximide show overall similarity but minor differences to the IE pattern (McDonald et al., manuscript in preparation).

Intracellular distribution and electrophoretic mobility of IE polypeptides. It has been reported that many IE polypeptides are translocated to the nucleus and that some undergo post-translational modification to forms of lower electrophoretic mobility (2, 4, 5, 16). These two features were examined in wt HSV-1- and *tsK*-infected cells. To obtain radiolabeled IE polypeptides, cells were infected in the presence of 200 μg of cycloheximide per ml and maintained at 38.5°C for 5 h. Cycloheximide was then removed by thoroughly washing the monolayers, and phosphate-buffered saline containing [^{35}S]methionine was added to the cultures for 25 min. The labeling medium was then replaced by growth medium containing cycloheximide, and cultures were harvested and fractionated into "nuclei" and "cytoplasm," and polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The design of the experiment is based on previous findings (18). The 25-min labeling period was chosen to be a time at which IE polypeptides were the only virus-induced species labeled and mRNA for later "non-IE" functions (e.g., dPyK) had not been synthesized. Furthermore, under the conditions of the "chase," it has been shown that production of dPyK mRNA occurs in all cases except in *tsK*-infected cells at 38.5°C. Therefore, this experiment examines the fate of the *ts* IE polypeptide in the functional (both viruses at 31°C, wt HSV-1 at 38.5°C) and nonfunctional (*tsK* at 38.5°C) states.

The method of cell fractionation used was developed for maximum reproducibility in handling large numbers of samples and does not necessarily represent the most rigorous means for separating nuclei and cytoplasm. Nevertheless, examination of polypeptides in nuclear and cytoplasmic fractions of mock-infected or infected cells shows that many species partition exclusively in one fraction, and these presumably represent tightly bound nuclear components and freely soluble cytoplasmic polypeptides. Those which appear in both fractions may represent nuclear contents which are eluted during cell lysis, cytoplasmic structures which remain partially attached to nuclei, or polypeptides which truly exist in both nuclei and cytoplasm. The description of fractions as nuclear or cytoplasmic is therefore made for convenience and may not reflect precise intracellular compositions.

Cytoplasmic fractions are shown in Fig. 2. After pulse-labeling, polypeptides V_{mw} 175, V_{mw} 136, V_{mw} 110, V_{mw} 63, and V_{mw} 12 were detected in both wt HSV-1- and *tsK*-infected cells. Upon further incubation at 31°C, the relative amounts of V_{mw} 175 and V_{mw} 110 decreased, but at 38.5°C a major difference between wt HSV-1- and *tsK*-induced IE polypeptides was observed: significant amounts of V_{mw} 175 were recovered in the cytoplasmic fractions of *tsK*-infected cells.

Figure 3 shows the nuclear fractions from this

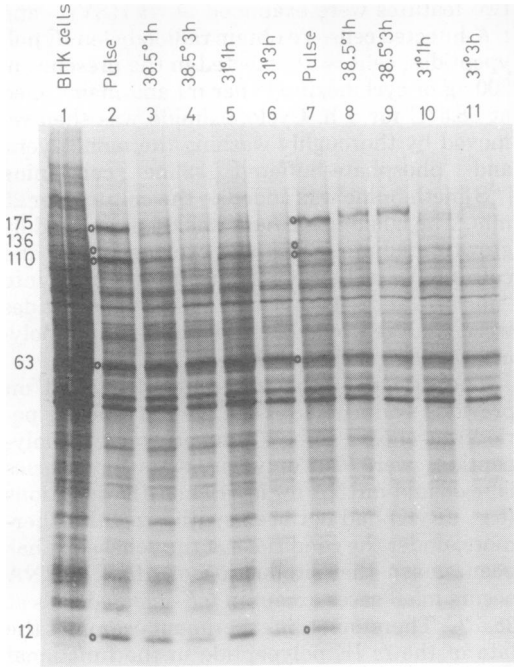

FIG. 2. *Analysis of cytoplasmic IE polypeptides. Cells were infected with wt HSV-1 or tsK at 38.5°C in the presence of cycloheximide and incubated for 5 h. Cycloheximide was then removed by washing, and cells were pulse-labeled with [^{35}S]methionine for 25 min. Labeling medium was then replaced with medium containing cycloheximide. Incubation was continued (chase), and cells were fractionated into cytoplasm and nuclei. Cytoplasmic fractions were analyzed on 9 to 18% gradient DATD-cross-linked polyacrylamide gels. wt HSV-1-infected cells were pulse-labeled (track 2) and chased for 1 h (track 3) or 3 h (track 4) at 38.5°C and 1 h (track 5) or 3 h (track 6) at 31°C. tsK-infected cells were pulse-labeled (track 7) and chased for 1 h (track 8) or 3 h (track 9) at 38.5°C and 1 h (track 10) or 3 h (track 11) at 31°C. Cytoplasmic polypeptides of mock-infected BHK cells are shown in track 1. IE polypeptides* (○) *are labeled to the left of track 1.*

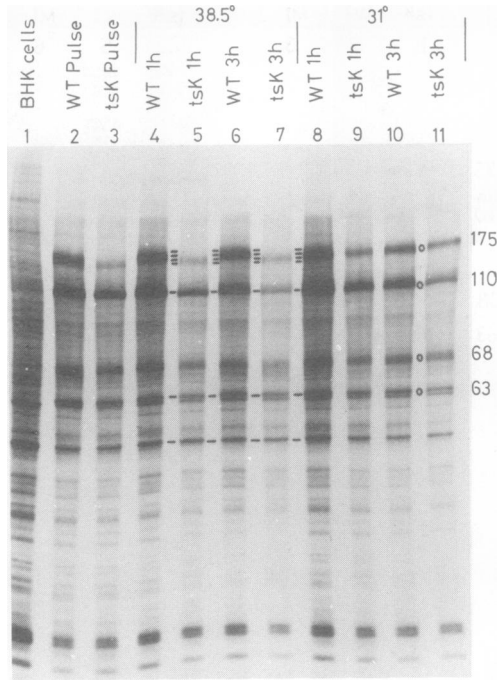

FIG. 3. *Analysis of nuclear IE polypeptides. Cells were infected and treated as described in the legend to Fig. 2, and nuclear fractions were analyzed on 9 to 18% gradient DATD-cross-linked gels. wt HSV-1-infected cells were pulse-labeled (track 2) and chased for 1 h (track 4) or 3 h (track 6) at 38.5°C and 1 h (track 8) or 3 h (track 10) at 31°C. tsK-infected cells were pulse-labeled (track 3) and chased for 1 h (track 5) or 3 h (track 7) at 38.5°C and 1 h (track 9) or 3 h (track 11) at 31°C. Nuclear polypeptides of mock-infected BHK cells are shown in track 1. IE polypeptides* (○) *are labeled to the right of track 11. To assist visualization of the mobility changes in V_{mw} 175, parallel lines (1 mm apart in the V_{mw} 175 region) have been drawn on the autoradiogram at appropriate positions.*

experiment. Comparison of pulse-labeled IE polypeptides of wt HSV-1 with mock-infected cell polypeptides shows the presence of V_{mw} 175, V_{mw} 110, V_{mw} 68, and V_{mw} 63. When nuclear pulse-labeled *tsK*-induced IE polypeptides were compared with those of wt HSV-1 (tracks 2 and 3), polypeptide V_{mw} 175 had an increased average electrophoretic mobility. Although small, this alteration could be demonstrated reproducibly. The difference was maintained during further incubation at 38.5°C (tracks 4 to 7), but at 31°C the electrophoretic mobility of *tsK*-induced V_{mw} 175 was indistinguishable from that of the wt HSV-1-induced polypeptide (tracks 8 to 11).

Quantitative analysis of IE polypeptides. The presence of V_{mw} 175 in cytoplasmic fractions of *tsK*-infected cells at 38.5°C could represent an abnormal partitioning during cell fractionation or a decreased rate of degradation of this polypeptide. To resolve these possibilities and to describe the distribution of IE polypeptides in a quantitative way, microdensitometer tracings of the autoradiograms shown in Fig. 2 and 3 were made (Fig. 4).

Visual comparison of the peak heights of a major cellular polypeptide of molecular weight 45,000, presumed to be actin, V_{mw} 63, and V_{mw} 175 suggests that relatively less nuclear V_{mw} 175 is present in *tsK*-infected cells at 38.5°C than at

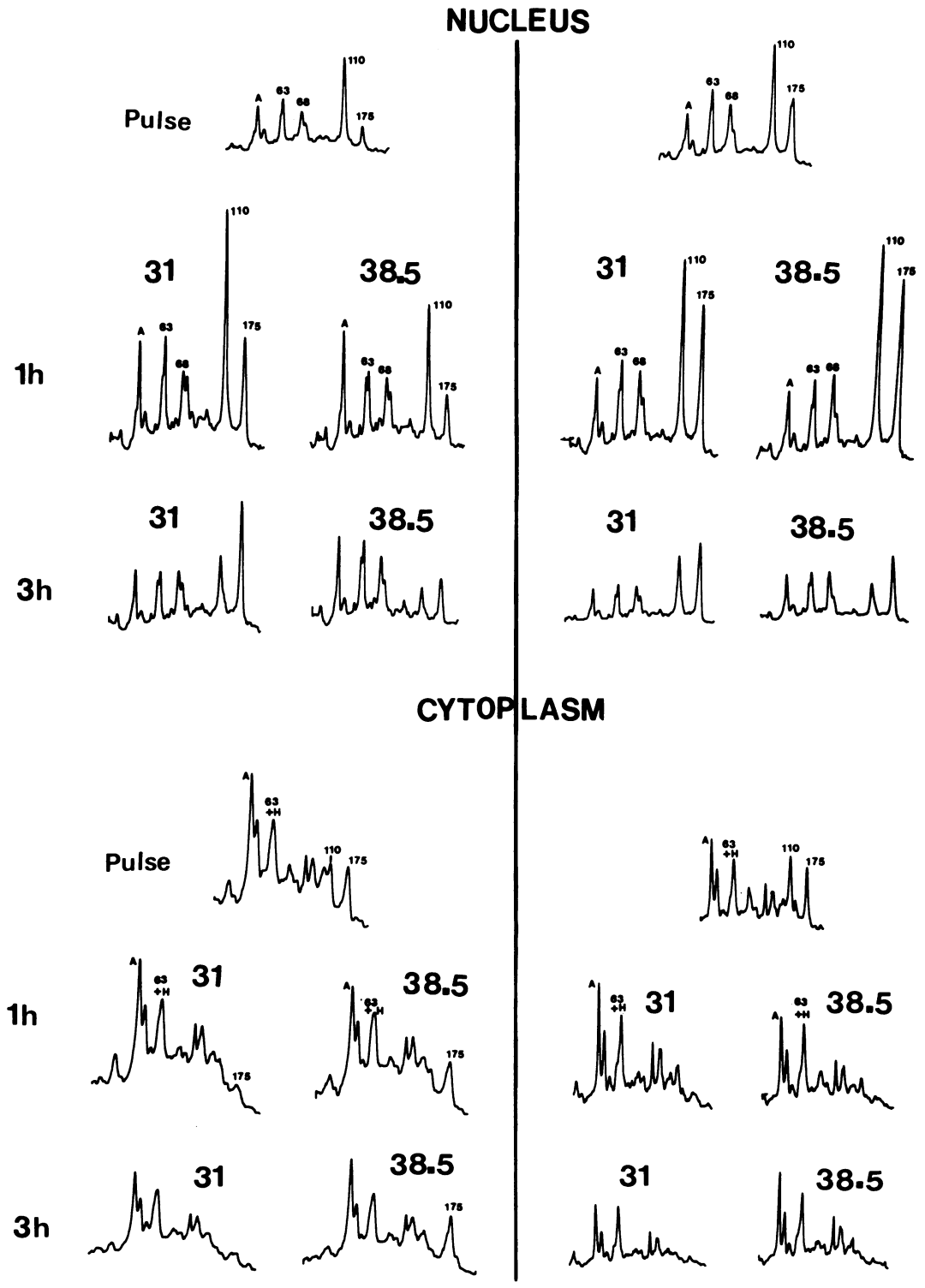

FIG. 4. *Microdensitometer tracings of the samples analyzed in Fig. 2 and 3. (Left) tsK; (right) wt.*

31°C, whereas such a difference does not hold for wt HSV-1-infected cells. The increased level of V_{mw} 175 in *tsK*-infected cell cytoplasm at 38.5°C is also clearly shown.

The results of estimating the radioactivity in three IE polypeptides, V_{mw} 175, V_{mw} 110, and V_{mw} 63, compared with actin, are shown in Table 1. Polypeptide V_{mw} 63 showed no significant change in distribution, compared with actin, between 31 and 38.5°C in both wt HSV-1- and *tsK*-infected cells. This result confirms the validity of the normalization procedure used and helps to define the errors in an individual experiment. Polypeptide V_{mw} 110 showed instability during the chase, but the magnitude of this effect, although generally more pronounced at 38.5°C, was not reproducible between experiments. No correlation between the instability of V_{mw} 110 and the virus preparation used (wt HSV-1 or *tsK*) could be demonstrated. Polypeptide V_{mw} 175 was consistently markedly reduced in intensity in nuclei of *tsK*-infected cells at 38.5°C. The increased level of V_{mw} 175 in the cytoplasm of *tsK*-infected cells was also demonstrated and, since 60% of the radioactive actin was recovered in the cytoplasmic fraction (see above), it can be calculated that this material fully accounted for the reduced nuclear V_{mw} 175 content.

Figure 5 shows pooled data from three separate experiments in which the percentage of radioactivity in IE polypeptides recovered in the nuclear fraction was calculated. The striking result is that approximately 40% of *tsK*-induced V_{mw} 175 was in the nuclear fraction at 38.5°C, whereas this figure increased to greater than 90% at 31°C. The behavior of V_{mw} 110 and V_{mw} 63 was indistinguishable between wt HSV-1- and *tsK*-infected cells.

TABLE 1. *Analysis of IE polypeptides*

Cell sample	Relative peak area[a]					
	V_{mw} 175		V_{mw} 110		V_{mw} 63	
	Nuc	Cyt	Nuc	Cyt	Nuc	Cyt
wt pulse	1.8	0.6	2.3	0.7	1.2	ND[b]
wt, 31°C, 1 h	2.8	0.1	2.9	0.3	1.5	ND
wt, 31°C, 3 h	3.3	0.0[c]	2.7	0.0	1.2	ND
wt, 38.5°C, 1 h	2.9	0.0	3.0	0.1	1.3	ND
wt, 38.5°C, 3 h	2.0	0.0	1.4	0.0	1.2	ND
tsK pulse	0.6	0.4	2.2	0.4	0.9	ND
tsK, 31°C, 1 h	1.6	0.1	2.5	0.3	0.9	ND
tsK, 31°C, 3 h	2.2	0.0	1.5	0.0	0.8	ND
tsK, 38.5°C, 1 h	0.6	0.4	1.6	0.1	0.9	ND
tsK, 38.5°C, 3 h	0.7	0.5	0.6	0.0	0.9	ND

[a] The peak areas of the bands shown in Fig. 4 are expressed relative to the peak area of actin (A). Nuc, Nucleus; Cyt, cytoplasm.

[b] ND, Not determined, since a major cytoplasmic BHK cell band comigrated with V_{mw} 63.

[c] Zero values represent <0.05.

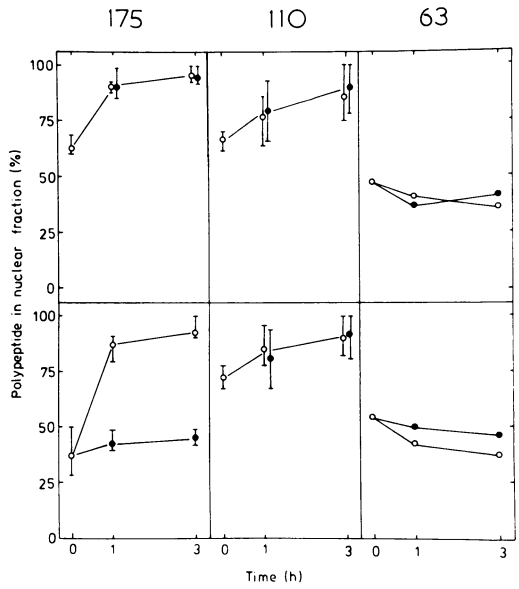

FIG. 5. *Intracellular distribution of IE polypeptides at 31°C (○) and 38.5°C (●). The percentage of the radioactive polypeptide recovered in the nuclear fraction was calculated as described in the text. The data for V_{mw} 175 and V_{mw} 110 show the means and range of values from three separate experiments. The data for V_{mw} 63 are taken from a single experiment in which this polypeptide was resolved clearly in the cytoplasmic fraction.*

Therefore, this analysis shows that V_{mw} 175 was stable during the chase period of 3 h, but that its distribution upon cell fractionation was abnormal in *tsK*-infected cells at 38.5°C.

Analysis of a *tsK* revertant. If the *ts* defects described above are related to the mutation affecting induction of dPyK mRNA and growth of the virus, it might be expected that revertants of *tsK* would show normal metabolism of V_{mw} 175.

The properties of V_{mw} 175 in cells infected with *tsK* or a spontaneous revertant of *tsK* are shown in Fig. 6. The revertant shows little recovery of V_{mw} 175 in the cytoplasmic fraction after incubation for 3 h at 38.5°C (track 6) and also shows normal electrophoretic mobility of this polypeptide at 38.5 and 31°C (tracks 11 and 13). The revertant therefore behaves as wt HSV-1 in the metabolism of V_{mw} 175.

Synthesis of IE polypeptides in mixedly infected cells. The experiments described above show that the IE Polypeptide V_{mw} 175 or *tsK* has two abnormal properties at 38.5°C. Relatively large amounts were recovered in the cytoplasm after cell fractionation, and the nu-

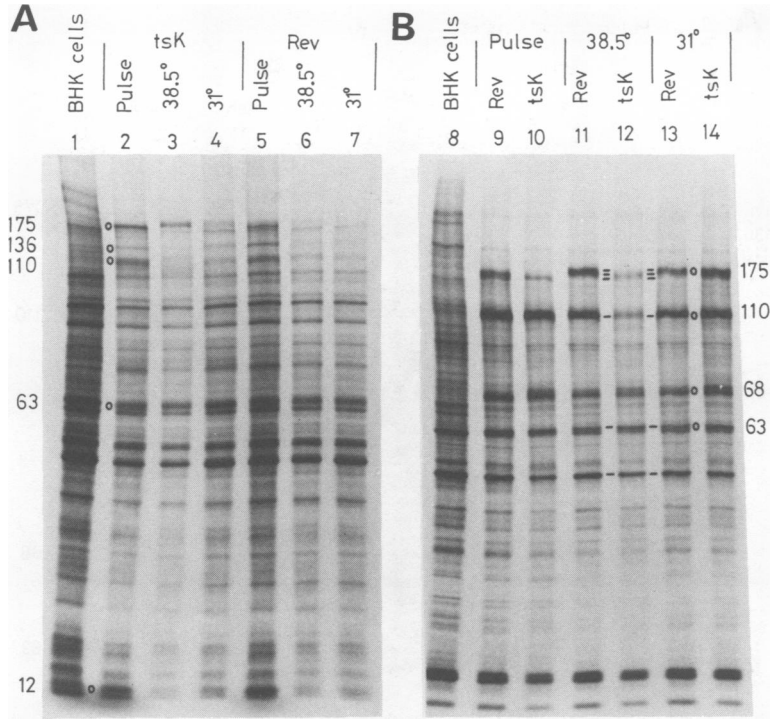

FIG. 6. *Analysis of IE polypeptides induced by a tsK revertant (Rev). Cells were infected with tsK or a revertant of tsK and treated as described in the legend to Fig. 2. Polypeptides were analyzed on 9 to 18% gradient DATD-cross-linked gels. (A) Cytoplasmic fractions of cells infected with tsK (tracks 2 to 4) or the revertant (tracks 5 to 7) show polypeptides after pulse-labeling (tracks 2 and 5) and incubation at 38.5°C (tracks 3 and 6) or 31°C (tracks 4 and 7). Track 1 shows mock-infected cell polypeptides. (B) Nuclear fractions of cells infected with tsK (tracks 10, 12, and 14) or the revertant (tracks 9, 11, and 13) show polypeptides after pulse-labeling (tracks 9 and 10) and incubation at 38.5°C (tracks 11 and 12) or 31°C (tracks 13 and 14). Track 8 shows mock-infected cell polypeptides. Reference lines assist visualization of the electrophoretic mobility differences of V_{mw} 175, as described in the legend to Fig. 3.*

clear form was not processed at normal rates. These changes were reversed at 31°C, therefore, a correlation between the presence of abnormal V_{mw} 175 and the failure to induce dPyK mRNA (18) exists.

The aberrant properties of V_{mw} 175 in *tsK*-infected cells at 38.5°C could be due to an alteration in the polypeptide itself or in another IE polypeptide whose function is to modify V_{mw} 175. These two possibilities were investigated in cells coinfected with wt HSV-1 and *tsK*. IE polypeptides were labeled and analyzed as described above. If the lesion affecting V_{mw} 175 were in the polypeptide itself, both wt and aberrant forms should be detectable during the chase at 38.5°C, but if the lesion were in a modifying IE polypeptide, it might be expected that the wt product would complement the defect.

Figure 7A shows cytoplasmic fractions from cells infected with *tsK* (40 PFU/cell), wt HSV-1 (40 PFU/cell), or a mixture of the two viruses (20 PFU of each per cell). As found previously, greater amounts of V_{mw} 175 were present in the cytoplasmic fraction of *tsK*-infected cells than in wt HSV-1-infected cells at 38.5°C (tracks 5 and 7) but not at 31°C (tracks 8 and 10). The mixedly infected cells also showed increased levels of V_{mw} 175 after incubation at 38.5°C (track 6), suggesting that the presence of wt IE polypeptides had not affected this aspect of the *tsK* lesion.

Analysis of the nuclear polypeptides (Fig. 7B) again showed that *tsK*-induced V_{mw} 175 had increased electrophoretic mobility at 38.5°C and that mixedly infected cells produced both wt and abnormal forms of this polypeptide. Therefore, it appears that this lesion was also unaffected by the presence of wt IE polypeptides.

Effects of temperature upshift on *tsK*- and wt HSV-1-infected cells. Previous experiments (18) showed that the functional lesion in *tsK*-infected cells could be reversed upon temperature downshift in the absence of further

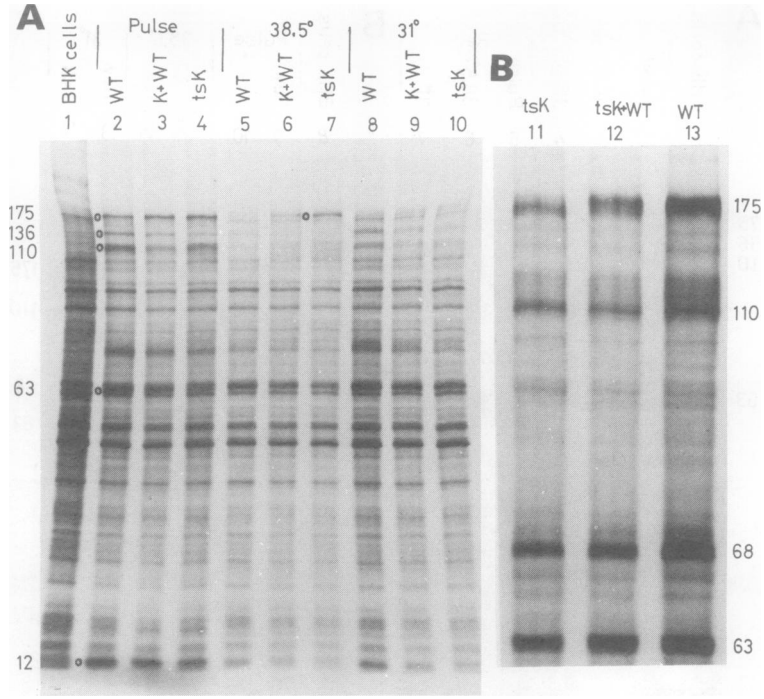

FIG. 7. *Analysis of IE polypeptides in mixedly infected cells. Cell cultures were infected with wt HSV-1 (40 PFU/cell [tracks 2, 5, and 8]), tsK (40 PFU/cell [tracks 4, 7, and 10]), or a mixture of the two viruses (20 PFU of each per cell [tracks 3, 6, and 9]) and treated as described in the legend to Fig. 2. (A) Cytoplasmic samples were analyzed on 9 to 18% gradient DATD-cross-linked gels. Pulse-labeled polypeptides are shown in tracks 2, 3, and 4; polypeptides present after a 3-h chase at 38.5°C are shown in tracks 5, 6, and 7; and polypeptides present after a 3-h chase at 31°C are shown in tracks 8, 9, and 10. Mock-infected cell polypeptides are shown in track 1. (B) A photographic enlargement of a 6 to 15% gradient gel of nuclear samples after incubation at 38.5°C shows polypeptides induced by tsK (track 11), wt HSV-1 (track 13), or a mixture of the two viruses (track 12).*

protein synthesis, and the results presented above demonstrate that the same is true for the aberrant properties of V_{mw} 175. Since it is throught that the functional lesion is at the transcriptional level (18, 24), it was considered important to determine whether *tsK*-infected cells reverted to the mutant phenotype after a temperature upshift from 31 to 38.5°C. The first experiment of this type was designed to investigate whether the aberrant properties of V_{mw} 175 were reversible.

Cells were infected with *tsK* in the presence of cycloheximide, and IE polypeptides were labeled as described above. Cycloheximide was readded, and cultures were maintained at 31 or 38.5°C. After 1 h, one culture was transferred from 31 to 38.5°C and harvested 2 h later; after a further 1 h, another culture was upshifted and harvested 1 h later. The distribution of IE polypeptides in nuclear and cytoplasmic fractions was examined. Figure 8 shows the presence of V_{mw} 175 in both cytoplasmic and nuclear fractions after the pulse-labeling period (tracks 2 and 15) and its retention in the cytoplasm at 38.5°C (tracks 7 and 8) but not at 31°C (tracks 3 and 4). The culture which was upshifted after 1 h (a time at which almost all V_{mw} 175 was recovered in the nuclear fraction [see track 3]) and maintained at 38.5°C for 2 h showed high levels of V_{mw} 175 in the cytoplasmic fraction (track 5) and increased electrophoretic mobility of nuclear V_{mw} 175 (track 12). Some cytoplasmic V_{mw} 175 was also detected after the upshift for 1 h after 2 h at 31°C (track 6).

This experiment, together with the data presented above (Fig. 2 and 3), shows that the two aberrant properties of *tsK*-induced V_{mw} 175 are reversible during temperature shifts between 31 and 38.5°C.

Reversibility of the function of the *tsK* lesion was investigated by analysis of new polypeptide production after resumption of protein synthesis in cycloheximide-blocked cells. Cultures were infected with *tsK* or wt HSV-1 at 38.5°C in the

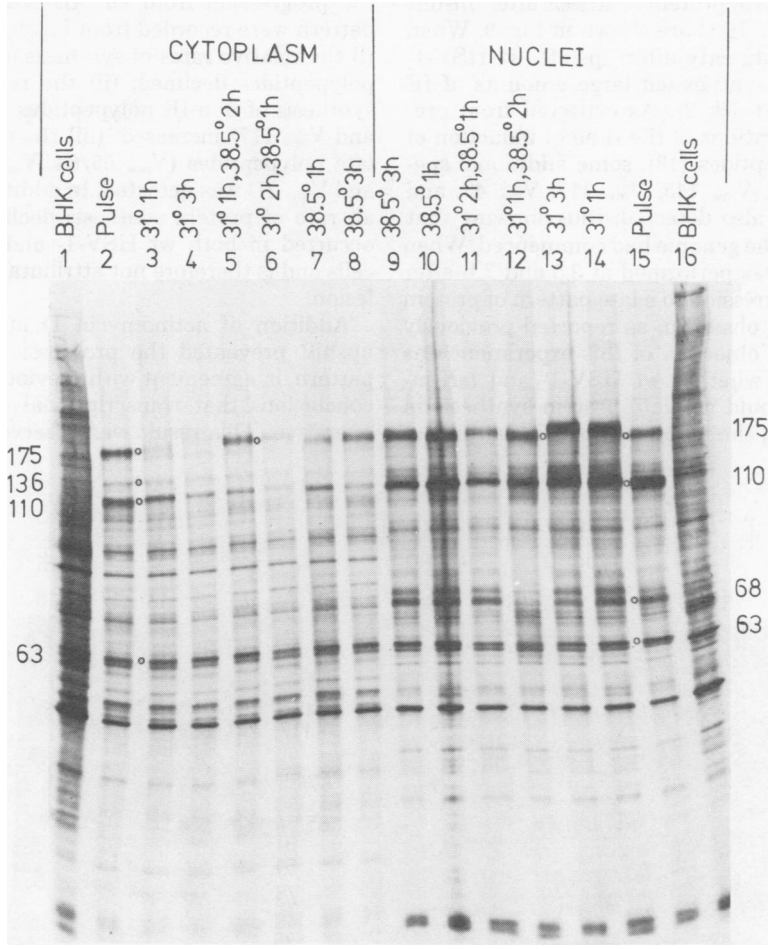

FIG. 8. *Reversibility of the properties of tsK IE polypeptides. Cell cultures were infected with tsK at 38.5°C in the presence of cycloheximide, and incubation was continued for 5 h. Cycloheximide was removed by washing, cells were pulse-labeled with [^{35}S]methionine for 25 min, and medium containing cycloheximide was added. One culture was harvested immediately, two were incubated at 38.5°C, and four were incubated at 31°C. Of the latter, one culture was transferred to 38.5°C after 1 h and harvested 2 h later; another was transferred to 38.5°C after 2 h and harvested 1 h later. Cytoplasmic and nuclear fractions were analyzed on a 6 to 15% gradient DATD-cross-linked gels, and dilutions were such that the amount of cytoplasmic material added was one half that of nuclear material. Cytoplasmic fractions are shown in tracks 1 to 8; nuclear fractions are shown in tracks 9 to 16. (tracks 2 and 15) Pulse-labeled polypeptides; (tracks 3 and 14) after 1 h at 31°C; (tracks 4 and 13) after 3 h at 31°C; (tracks 5 and 12) after 1 h at 31°C, followed by 2 h at 38.5°C; (tracks 6 and 11) after 2 h at 31°C, followed by 1 h at 38.5°C; (tracks 7 and 10) after 1 h at 38.5°C; (tracks 8 and 9) after 3 h at 38.5°C; (tracks 1 and 16) mock-infected cells.*

presence of cycloheximide; after 5 h, the inhibitor was removed and the monolayers were washed. Cultures were then downshifted to 31°C and incubated for 45 min. During this time, activation of later (non-IE) genes, including dPyK, occurs (18). After this period, cells were shifted back to 38.5°C, and incubation was continued with or without the addition of actinomycin D. A control, in which cultures after the removal of cycloheximide were incubated at 31°C throughout, was also included. The progression of viral protein synthesis was investigated by pulse-labeling in the presence of actinomycin D at various times after upshift. Addition of actinomycin D at the time of upshift was a control to demonstrate the effects of instantly inhibiting RNA synthesis. The inhibitor was added during pulse-labeling to prevent progression of the protein synthesis program during the labeling period.

The patterns of protein synthesis after upshift after 45 min at 31°C are shown in Fig. 9. When pulse-labeled directly after upshift, wt HSV-1-infected cells synthesized large amounts of IE polypeptides (track 2). As expected from previous considerations of the time of induction of non-IE polypeptides (18), some additional species (V_{mw} 155, V_{mw} 145, V_{mw} 117, V_{mw} 43, and V_{mw} 38) were also detected, thus showing that activation of the genome had commenced. When the labeling was performed at 3.5 and 7 h after upshift, a progression to a late pattern of protein synthesis was observed, as reported previously (7). Since the objective of this experiment was to determine whether wt HSV-1- and *tsK*-infected cells could activate protein synthesis in the same way, the following specific features of the progression from an "IE-like" to a "late" pattern were recorded from Fig. 9, tracks 2 to 4: (i) the relative rates of synthesis of IE and host polypeptides declined; (ii) the relative rate of synthesis of non-IE polypeptides (e.g., V_{mw} 155 and V_{mw} 117) increased; (iii) the appearance of late polypeptides (V_{mw} 65/64, V_{mw} 51, V_{mw} 40, and V_{mw} 21) was detected. In addition, the overall rate of protein synthesis declined, but this occurred in both wt HSV-1- and *tsK*-infected cells and is therefore not attributable to the *tsK* lesion.

Addition of actinomycin D at the time of upshift prevented the progression to the late pattern, in agreement with previously published conclusions that transcriptional controls were operative. This result was observed in both wt

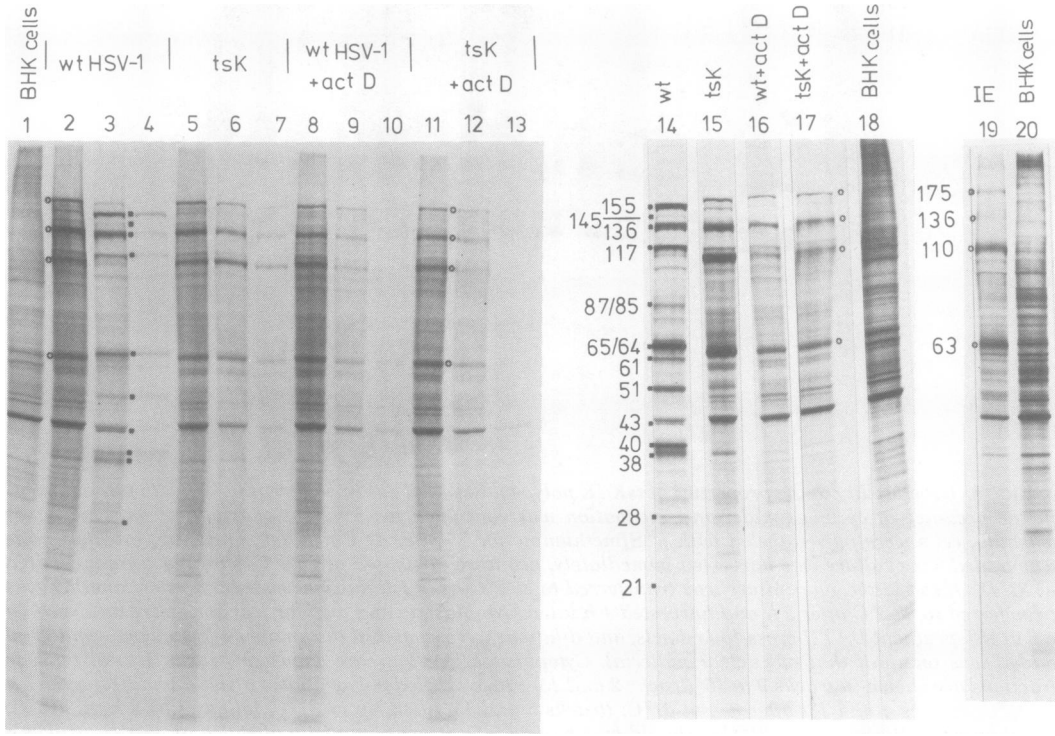

FIG. 9. *Reversibility of the tsK lesion. Cells were infected with wt HSV-1 or tsK at 38.5°C in the presence of cycloheximide and incubated for 5 h. Cycloheximide was then removed by washing, and cells were transferred to 31°C. After 45 min, cultures were upshifted to 38.5°C and divided into two sets. One set was treated with actinomycin D (act D) at the time of upshift. At various times after upshift, cultures were pulse-labeled for 30 min with medium containing [^{35}S]methionine and actinomycin D. Whole-cell samples were analyzed on 6 to 15% gradient MBA-cross-linked gels. Tracks 1 and 18 show mock-infected cells; tracks 2 to 4 show wt HSV-1-infected cells; tracks 5 to 7 show tsK-infected cells; tracks 8 to 10 show actinomycin D-treated wt HSV-1-infected cells; and tracks 11 to 13 show actinomycin D-treated tsK-infected cells. The labeling times were 0 to 0.5 h after upshift (tracks 2, 5, 8, and 11), 3.5 to 4 h after upshift (tracks 3, 6, 9, and 12), or 7 to 7.5 h after upshift (tracks 4, 7, 10, and 13). Longer exposures of tracks 4, 7, 10, and 13 are shown in tracks 14, 15, 16, and 17, respectively. Track 19 shows HSV-1 IE polypeptides compared with uninfected cell polypeptides (track 20). IE polypeptides (○) are labeled between tracks 18 and 19, and non-IE polypeptides (■) are labeled between tracks 13 and 14.*

HSV-1 (tracks 8 to 10)- and *tsK* (tracks 11 to 13)-infected cells.

Upshift of *tsK*-infected cells without actinomycin D did not give the progressive change in protein synthesis found with wt HSV-1-infected cells (tracks 5 to 7) but was similar to that found when RNA synthesis was inhibited. None of the three features tabulated above was observed, but the relative rate of synthesis of the IE polypeptide V_{mw} 110 increased. Therefore, upshift of *tsK*-infected cells affects the synthesis of non-IE polypeptides in the same way as does the total inhibition of RNA synthesis, even though activation of the relevant genes had occurred during the 45-min period at 31°C. This result shows that activation of the HSV genome is blocked in *tsK*-infected cells at 38.5°C, and the defective polypeptide is normally required in a functional state for a considerable time.

When cultures were maintained at 31°C (Fig. 10), both HSV-1- and *tsK*-infected cells showed the three features of protein synthesis progression described above, demonstrating that reversal of the *tsK* lesion could occur.

The experiment described above (Fig. 9) was also performed after 90 min at 31°C (data not shown). Although, due to the longer period at 31°C, more extensive activation of the virus genome occurred, upshift of *tsK*-infected cells to 38.5°C still gave an effect equivalent to adding actinomycin D. Thus, although a substantial progression to a non-IE pattern had occurred, the IE polypeptide containing the *tsK* mutation was still required in a functional state for the transcription of many non-IE genes.

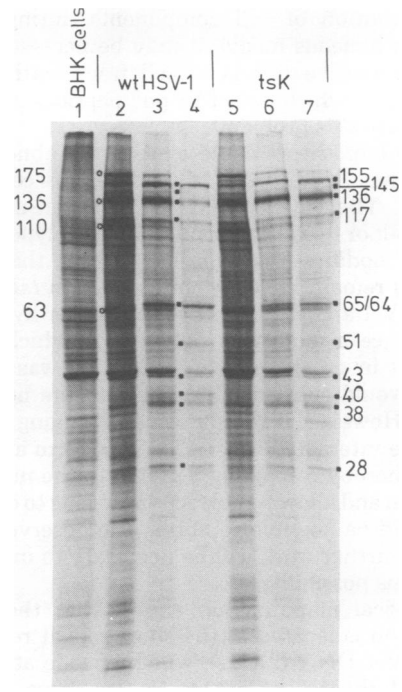

FIG. 10. *Induction of non-IE polypeptides at 31°C. Cells were infected and treated as described in the legend to Fig. 9. After 45 min at 31°C, cells were maintained at 31°C instead of being unshifted, and no actinomycin D-treated cells were analyzed. Track 1 shows mock-infected cells; tracks 2 to 4 show wt HSV-1-infected cells; and tracks 5 to 7 show tsK-infected cells. The labeling times were 0.75 to 1.25 h (tracks 2 and 5), 4.25 to 5.0 h (tracks 3 and 6), or 7.75 to 8.5 h (tracks 4 and 7) after removal of cycloheximide. IE polypeptides (○) are labeled to the left of track 1, and non-IE polypeptides (■) are labeled to the right of track 7.*

DISCUSSION

The results presented here show two aberrant properties of V_{mw} 175 in *tsK*-infected cells: impaired migration to the nucleus and failure to be processed normally. These properties can be correlated with a failure to induce dPyK mRNA (18) or to maintain the synthesis of many non-IE mRNA's (Fig. 9), and provide a basis for an investigation of HSV-1 transcriptional controls. It is not clear whether the *ts* defects in migration and processing of V_{mw} 175 are the direct causes of the transcriptional lesion, or additional manifestations of the *tsK* mutation, but certainly the transcriptional defect cannot be caused entirely by impaired migration of V_{mw} 175, since a considerable amount of this polypeptide was found in nuclei of *tsK*-infected cells at 38.5°C.

The processing of V_{mw} 175 is abnormal in *tsK*-infected cells at 38.5°C, as characterized by a failure to achieve the normal increase in apparent molecular weight during a 3-h period. This result cannot be fully interpreted at present, since the structural basis for the changes in mobility of V_{mw} 175 is uncertain. An obvious possibility is successive phosphorylation events, but is should be noted that all forms are labeled with [^{32}P]phosphate (16), as is V_{mw} 175 in *tsK*-infected cells at 38.5°C (D. McDonald, personal communication). If phosphorylation is involved, then a dephosphorylation mechanism must also exist, since reversal to higher electrophoretic mobility occurs upon temperature upshift (Fig. 8).

After a short labeling period and incubation at 38.5°C, V_{mw} 175 was recovered in the cytoplasm of *tsK*-infected cells, in contrast to wt HSV-1-infected cells. It is not clear whether this represents the true intracellular location or whether the polypeptide is more loosely associated with nuclear components and hence readily eluted during cell lysis. In view of the possible

redistribution of cell components during cell lysis in aqueous media, it may be necessary to use alternative means of cell fractionation or techniques which detect IE polypeptides in vivo to resolve this problem.

Two hypotheses could explain the abnormal properties of V_{mw} 175 in *tsK*-infected cells at 38.5°C. They might be due to a lesion in V_{mw} 175 itself or to a lesion in another IE polypeptide which modifies V_{mw} 175. The finding that the defects remain in cells coinfected with *tsK* and wt HSV-1 argues in favor of the former hypothesis, since a modifying polypeptide which was present in excess, or whose function was catalytic, would convert all V_{mw} 175 to the normal form. However, if the putative modifying polypeptide interacted with V_{mw} 175 to form a complex, thereby restricting V_{mw} 175 to the nuclear fraction and allowing correct processing to occur, it would be possible to obtain the observed results. Further work will be necessary to investigate this possibility.

Physical mapping has shown that the *tsK* mutation is located in the short repeat regions of HSV-1 DNA (21, 22), which encode at least part of the V_{mw} 175 (14, 15, 19). Two other mutants of strain 17, *tsD* and *tsT*, are located near *tsK* and show many similarities in the polypeptides detected at 38.5°C, either by pulse-labeling or by in vitro translation, at 5 h postinfection (C. Preston, unpublished data). In particular, IE polypeptides are overproduced, and V_{mw} 175 shows the same increased electrophoretic mobility (MacDonald et al., manuscript in preparation) and aberrant distribution upon cell fractionation (Preston, unpublished data) as does *tsK*. The RNA species synthesized in *tsK*-, *tsD*-, and *tsT*-infected cells are also very similar (24), but limited amounts of some non-IE polypeptides are induced by *tsD* and *tsT* but not *tsK*.

Two approaches followed by other groups are relevant to the results presented here. Recent studies have shown that the Houston mutant *tsB2* fails to activate the endogenous HSV dPyK gene of HSV-1-transformed cells at the nonpermissive temperature and is therefore similar to *tsK* (9). Interestingly, these mutants share some other biochemical properties, since *tsB2* shows similarities to the *tsK* polypeptide profile, particularly enhanced synthesis and aberrant processing of V_{mw} 175 (2, 3). Treatment of cells with the arginine and analog canavanine also gives a polypeptide profile similar to that of *tsK*-infected cells (8) and causes impaired transport of IE polypeptides to the nucleus and inefficient processing of V_{mw} 175 (16).

The finding that upshift of *tsK*-infected cells reverses the migration and processing of V_{mw} 175 and arrests the normal program of virus protein synthesis suggests that V_{mw} 175 is required in a functional state for a considerable period of the growth cycle, rather than transiently. This polypeptide is presumably intimately associated with a transcriptional complex and may be able to alter the specificity of RNA synthesis by interaction with cellular RNA polymerase II or other components of such a complex. This represents a unique system in the study of eucaryotic cells. A polypeptide whose properties can be correlated with a transcriptional control has been identified, it can be labeled efficiently, and a mutant with a *ts* lesion affecting the polypeptide is available. An investigation of the biochemical properties of V_{mw} 175 is in progress.

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