JOURNAL OF VIROLOGY, Jan. 1974, p. 140-145
Vol. 13, No. 1

Printed in U.S.A.

# Virus Type-Specific Thymidine Kinase in Cells Biochemically Transformed by Herpes Simplex Virus Types 1 and 2

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Received for publication 23 August 1973

Transformation of mouse cells (Ltk$^-$) and human cells (HeLa Bu) from a thymidine kinase (TK)-minus to a TK$^+$ phenotype (herpes simplex virus [HSV]-transformed cells) has been induced by infection with ultraviolet-irradiated HSV type 2 (HSV-2), as well as by HSV type 1 (HSV-1). Medium containing methotrexate, thymidine, adenine, guanosine, and glycine was used to select for cells able to utilize exogenous thymidine. We have determined the kinetics of thermal inactivation of TK from cells lytically infected with HSV-1 or HSV-2 and from HSV-1- and HSV-2-transformed cells. Three hours of incubation at 41 C produces a 20-fold decrease in the TK activity of cell extracts from HSV-2-transformed cells and Ltk$^-$ cells lytically infected with HSV-2. The same conditions produce only a twofold decrease in the TK activities from HSV-1-transformed cells and cells lytically infected with HSV-1. This finding supports the hypothesis that an HSV structural gene coding for TK has been incorporated in the HSV-transformed cells.

L cells lacking thymidine kinase (Ltk$^-$ cells) can stably acquire the ability to synthesize thymidine kinase (TK) due to infection with ultraviolet-irradiated herpes simplex virus type 1 (HSV-1) (3, 13). Such cells will be referred to as HSV-1-transformed cells. The HSV-1-transformed cells can be distinguished from the parental Ltk$^-$ cells by the following criteria. (i) They have the ability to utilize exogenous thymidine for cellular DNA synthesis. (ii) They exhibit a 15- to 21-fold increase in TK activity relative to the low background activity present in Ltk$^-$ cells.

Cells lytically infected with HSV-1 exhibit a new TK activity which is not present in uninfected cells. This new TK activity has been distinguished from the TK activity present in uninfected cells by serological methods and by $K_m$ measurements (1, 2, 9).

We have demonstrated that the TK activity present in HSV-1-transformed cells is different from the TK activity present in normal L cells with respect to migration during electrophoresis in polyacrylamide gels (12). The TK activity induced during the course of lytic infection is electrophoretically indistinguishable from TK activity found in the HSV-1-transformed cells.

The origin of the new TK activity in the HSV-1-transformed cells could be explained by either of the following hypotheses. (i) HSV-1 carries a structural gene, coding for TK, which is incorporated into the genome of the HSV-1 transformed cells. (ii) Infection of Ltk$^-$ cells by ultraviolet-irradiated HSV-1 is able to cause a permanent derepression of an L-cell TK activity which is not normally expressed in tissue culture.

HSV type 2 (HSV-2) is the genital variant of herpes simplex. The TK induced during lytic infection by HSV-2 is serologically different from the TK induced by HSV-1 (17). In addition, the TK activity induced by HSV-2 is highly thermolabile relative to the HSV-1-induced TK activity (18).

To further investigate the origin of the new TK in HSV-transformed cells, we have transformed Ltk$^-$ cells with HSV-1 and HSV-2. In addition, we have transformed a 5-bromodeoxyuridine-resistant human cell line (HeLa Bu) with HSV-1 and HSV-2. If the new TK present in HSV-transformed cells is a viral gene product, L cells transformed with HSV-2 should express a new TK which is highly thermolabile relative to the TK present in the HSV-1-transformed L cells. Furthermore, the same difference in thermolability should be observed by using TK from HeLa Bu cells which have been transformed by HSV-1 and by HSV-2.

## MATERIALS AND METHODS

**Virus.** HSV-1 was obtained from D. Yohn, and HSV-2 (strain 333) was originally obtained from W. Rawls. Virus stocks were prepared as described previously (13), except that viruses were grown in monolay-

ers of human embryonic lung cells.

**Cell lines.** The cell lines used and supplements added to the culture medium for routine subculture are listed in Table 1. All cell lines were subcultured by trypsinization every 72 h for a minimum of 100 passages, and grown at 37 C in a humidified atmosphere consisting of 10% $CO_2$ and 90% air.

**Medium.** Eagle medium (6) containing nonessential amino acids, and supplemented with 5% calf serum for L-cell lines and with 10% calf serum for HeLa lines, was used for cell culture. Other additions were made as noted in Table 1.

**Cell transformation.** HSV-transformed cells were obtained by the procedure of Munyon et al. (13). Briefly, $Ltk^-$ or HeLa Bu cells were plated ($2 \times 10^6$ cells per petri dish; Falcon 6-cm diameter plastic dish) and incubated overnight in Eagle medium supplemented with 10% calf serum. After removal of the medium, the cells were infected with a 0.2-ml inoculum of ultraviolet-irradiated HSV-1 or HSV-2 and incubated for 1 h. The virus inoculum contained from $5 \times 10^6$ to $2.5 \times 10^7$ PFU/ml before irradiation and was irradiated to a survival of approximately $10^{-6}$. Unabsorbed virus was aspirated, and medium containing 5% (L cells) or 10% (HeLa Bu cells) calf serum was added. The dishes were incubated for 24 h, after which the Eagle medium was replaced by $TK^+$ selective medium (Eagle medium containing 5% calf serum, methotrexate, thymidine, adenosine, guanosine, and glycine [see Table 1]). This medium does not permit growth of $Tk^-$ cells but will allow the growth of $TK^+$ cells. After 20 days of incubation (37 C), about 15 colonies per dish appeared in the $Ltk^-$ dishes. Of these, five colonies per dish were in various stages of disintegration and were not subsequently viable. At this time, in the HeLa Bu plates about 20 colonies per dish appeared, but these were too small for isolation (<50 cells). The HeLa Bu dishes were refed with $TK^+$ selective medium and incubated a further 20 days. An average of about 10 colonies per dish remained. The colonies were of variable size, ranging from 50 to 500 cells per colony. The smaller colonies consistently disintegrated upon further incubation, and we therefore concluded that they had lost their ability to utilize exogenous thymidine. An average of one colony per dish continued to grow, and these could be isolated and subcultured indefinitely in selective medium.

In general, uninfected HeLa Bu cells died more slowly in selective medium than did $Ltk^-$ cells. Petri dishes ($2 \times 10^6$ cells per dish) were seeded with HeLa Bu and $Ltk^-$ cells and infected with identical virus inoculums (either HSV-1 or HSV-2). The frequency of occurrence of stably transformed HeLa Bu colonies was approximately 10-fold lower than the corresponding frequency of transformation of $Ltk^-$ cells.

**Enzyme preparation.** Soluble extracts of TK activity were prepared as follows. Monolayers ($5 \times 10^7$ cells) were washed twice in Eagle medium, scraped, and pelleted at $200 \times g$ for 15 min. Each pellet was suspended in 5 volumes of a buffer containing 0.01 M Tris-maleate (pH 6.5), 0.15 M KCl, 0.02 M MgCl, and 0.001 M mercaptoethanol. The cell suspensions were disrupted by three cycles of freeze-thawing. The cell lysates were then centrifuged at $130,000 \times g$ for 1 h, and the supernatants were generally stored overnight at −70 C. The amount of enzyme inactivation after 2 weeks of storage at −70 C was not reproducible, but in no case was it greater than 50%. The differential thermostability of HSV-1 and HSV-2 TKs was exhibited in both fresh enzyme preparations and in enzyme preparations which had been stored for up to 2 weeks.

**Tk assay.** The standard assay mixture contained the following final concentrations of solutes: 0.1 M maleate Tris (pH 6.5), 0.01 M ATP, 0.02 M $MgCl_2$, and $10^{-5}$ M [$H^3$]thymidine at a specific activity of 11 Ci/mmol. For thermal inactivation experiments, 0.1 ml of enzyme extract containing approximately 40 μg of protein was added to 0.1 ml of the assay mixture. The reaction mixture was incubated at 35 C for 15 min with shaking. A 0.25-ml volume of the reaction mixture was spotted in duplicate on 1-cm squares of diethylaminoethyl (DEAE)-cellulose. After the squares were washed three times in ammonium formate buffer (0.001 M, pH 3.6), two times in distilled water, and two times in 90% ethanol and dried,

TABLE 1. *Cell lines*

| Abbreviation | Reference | Phenotype | Supplements added to medium[a] |
|---|---|---|---|
| $L_{tk}$ | 16 | $Ltk^+$ | None |
| $Ltk^-$ | 9 | $Ltk^-$ | Budr[b] |
| Clone 111 | 13 | LHSV-1 transformed ($Tk^+$) | MTAGG[c] |
| Clone 120 | 13 | LHSV-1 transformed ($Tk^+$) | MTAGG |
| Clone 139 | 13 | LHSV-1 transformed ($Tk^+$) | MTAGG |
| Clone 202 | Davis, this publication | LHSV-2 transformed ($Tk^+$) | MTAGG |
| Clone 207 | Davis, this publication | LHSV-2 transformed ($Tk^+$) | MTAGG |
| Clone 208 | Davis, this publication | LHSV-2 transformed ($Tk^+$) | MTAGG |
| HeLa Bu | 10 | Budr-resistant HeLa ($Tk^-$) | Budr |
| Clone H101 | Davis, this publication | HeLa HSV-1 transformed ($Tk^+$) | MTAGG |
| Clone H201 | Davis, this publication | HeLa HSV-2 transformed ($Tk^+$) | MTAGG |

[a] The basic medium used for cell culture is Eagle minimal essential medium containing 5% calf serum.
[b] 5-Bromodeoxyuridine at a final concentration of 20 μg/ml.
[c] The additions to Eagle medium indicated by MTAGG are as follows: methotrexate, $6 \times 10^{-7}$ M; thymidine, $1.6 \times 10^{-5}$ M; adenosine, $5.0 \times 10^{-5}$ M; guanosine, $5 \times 10^{-5}$ M; glycine, $10^{-4}$ M.

radioactivity was determined in a liquid scintillation counter.

The $L_b$ enzyme exhibits a broad, flat pH optimum between pH 6.5 and 8.0. TK from HSV-1- and HSV-2-transformed cells and from cells lytically infected by these viruses has been assayed at pH 6.5 and at 8.0. The rate of conversion of thymidine to thymidine monophosphate is approximately the same for each enzyme at each pH.

**Thermal inactivation.** A 0.5-ml amount of an enzyme preparation in a stoppered, plastic test tube was placed in a 41.5 C constant temperature water bath. Samples were withdrawn after designated incubation times and assayed for residual thymidine kinase activity.

**Electrophoresis.** Polyacrylamide gel electrophoresis of TK activity was performed as described in Munyon et al. (12).

## RESULTS

**Thermal inactivation.** The TK activities of HSV-transformed L cells, Ltk$^-$ cells lytically infected with HSV-1 or HSV-2, and normal L cells (L cells containing their original TK activity) were compared with respect to their kinetics of thermal inactivation (Fig. 1). Enzyme extracts were incubated at 41.5 C, and the residual TK activity was determined for each preparation after 30, 60, and 180 min. At an incubation time of 180 min, the TK activity from HSV-2-transformed cells and Ltk$^-$ cells lytically infected with HSV-2 decreased 20-fold with respect to the activity at time zero. In contrast, the TK activities from HSV-1-transformed cells, Ltk$^-$ cells lytically infected with HSV-1, and normal L cells decreased less than twofold.

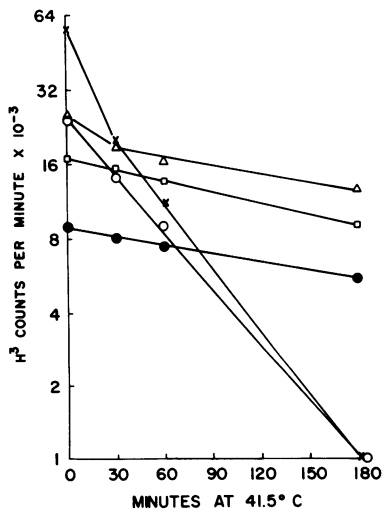


FIG. 1. *Kinetics of thermal inactivation of thymidine kinase from the following sources: clone 139 (Δ), Ltk$^-$ cells lytically infected with HSV-1 (□), clone 207 (○), Ltk$^-$ cells lytically infected with HSV-2 (×), and $L_b$ cells (●). Thymidine kinase activity is expressed as $^3H$ counts converted from Tdr to TMP. The preparation of enzyme extracts and thermal inactivation were performed as described in Materials and Methods.*

Experiments were then carried out to determine whether the difference in the thermostability of the TK activities from HSV-1- and HSV-2-transformed cells was a general property of the TKs from those two sources. Enzyme extracts were prepared from three clones of HSV-1- and three clones of HSV-2-transformed L cells. The thermostability of these TK activities were compared with TK activities induced during lytic infection of Ltk$^-$ cells by HSV-1 and HSV-2 (Table 2). The HSV-2 lytic TK activity and the TK activities from all of the HSV-2-transformed clones were highly thermolabile (approximately 1% residual activity) relative to the TK activities derived from all of the HSV-1-transformed clones and the HSV-1-induced lytic enzyme (greater than 50% residual activity).

Experiments were performed to determine whether the thermolabilities of the HSV-induced TK activities were affected by the cell type in which they were produced. HeLa cells (HeLa Bu) that died in TK$^+$ selective medium were transformed by HSV-1 or by HSV-2. Enzyme extracts were prepared and the kinetics of thermal inactivation was determined as described previously. The kinetics of thermal inactivation of TK derived from HSV-1- and HSV-2-transformed HeLa cells is shown in Fig.

TABLE 2. *Residual thymidine kinase activity after 0 and 180 min of incubation at 41.5 C*[a]

| Source of thymidine kinase | Enzyme | | P Survival (%) |
|---|---|---|---|
| | 0 min | 180 min | |
| Ltk$^-$ HSV-1 (lytic)[b] .. | 26,200 | 12,600 | 50 |
| Ltk$^-$ HSV-2 (lytic) .. | 56,500 | 100 | 1 |
| Cl 139 HSV-1 (tr)[c] ... | 16,900 | 9,100 | 54 |
| Cl 207 HSV-2 (tr) .... | 24,400 | 100 | 1 |
| Cl 111 HSV-1 (tr) .... | 14,000 | 14,300 | 100 |
| Cl 202 HSV-2 (tr) .... | 3,500 | 100 | 4 |
| Cl 120 HSV-1 (tr) .... | 7,700 | 6,700 | 87 |
| Cl 208 HSV-2 (tr) .... | 19,800 | 100 | 1 |
| $L_b$ ..... | 9,000 | 5,500 | 61 |

[a] Enzyme activity expressed as $^3H$ counts converted from Tdr to TMP during the thymidine kinase assay.

[b] Ltk$^-$ cells lytically infected with the indicated virus.

[c] tr, TK activity obtained from a clone of cells which had been transformed by the indicated virus.

2. The TK activity from the HSV-2-transformed cells was again highly thermolabile relative to the TK activity from the HSV-1-transformed cells.

Thouless and Skinner (18) reported that the TK lytically induced during HSV-2 infection is not protected from thermal inactivation by thymidine. We carried out experiments to determine the effect of $10^{-4}$ M thymidine on the thermostability of the TK activities from HSV-1- and HSV-2-transformed cells. At 41.5 C, thymidine at this molarity had no significant effect on the stability of either type of enzyme.

TK activity from HSV-2-transformed cells was not stabilized by a cell extract prepared from HSV-1-transformed cells that had been inactivated by heating at 56 C for 15 min. This heat treatment was sufficient to inactivate the HSV-1 TK activity. Conversely, HSV-2-transformed cell extract which had been inactivated at 56 C for 15 min did not increase the thermolability of the TK activity from HSV-1-transformed cells.

Therefore, the difference in thermostability of the TK activities from HSV-1- and HSV-2-transformed cells cannot be explained by the presence of a heat-stable substance in the enzyme extracts.

**Polyacrylamide gel electrophoresis.** The rate of migration during polyacrylamide gel electrophoresis was determined for the TK activities from $L_b$ cells lytically infected with HSV-1 and HSV-2, and from HSV-1 (cl 139)- and HSV-2 (cl 202)-transformed L cells. The $R_f$ of the peak activity for the HSV-2-infected cells was not significantly different from the $R_f$ of the TK peak from HSV-1-infected cells (Fig. 3). Similarly, there was no significant difference between the $R_f$ of the TK peaks from HSV-1- and HSV-2-transformed cells (Fig. 4). In a previous study (12) of the electrophoretic migration of TK from HSV-1-transformed cells and from cells lytically infected with HSV-1, the reproducibility of the position of the peaks in TK activity was only within 10% of the length

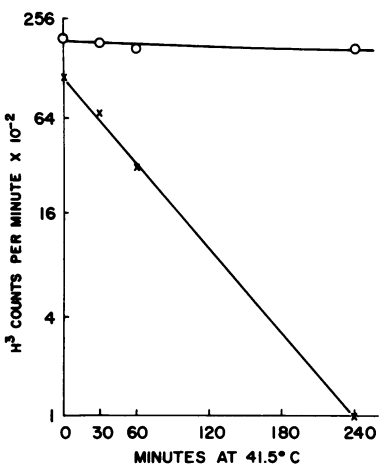


FIG. 2. *Kinetics of thermal inactivation of thymidine kinase from HeLa Bu cells transformed by HSV-1 and HSV-2. Enzyme extracts prepared from clH101* (O) *and CL H201* (×) *were subjected to thermal inactivation as described in Materials and Methods. Thymidine kinase activity is expressed as counts of ³H TMP converted from ³H Tdr in the assay mixture.*

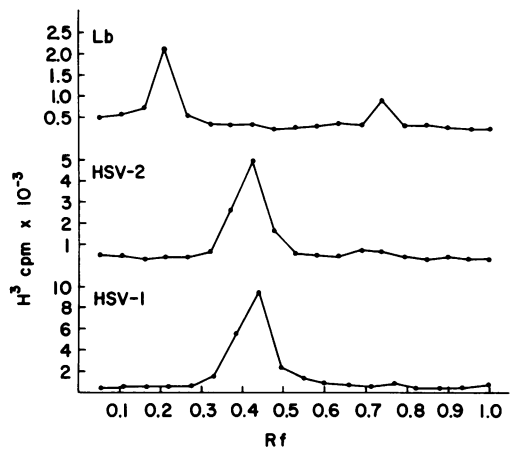


FIG. 3. *Polyacrylamide gel electrophoresis of TK activity from $L_b$ cells lytically infected with HSV-1 and HSV-2. Electrophoresis was performed as in Munyon et al. (12). $R_f$ is distance along the separating gel relative to the position of a bromophenol blue dye marker.*

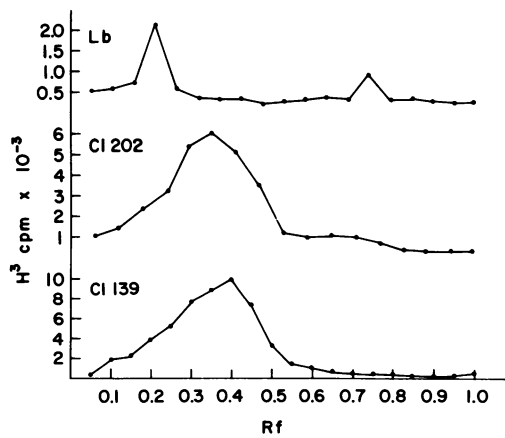


FIG. 4. *Polyacrylamide gel electrophoresis of TK activity from HSV-1 (cl 139)- and HSV-2 (cl 202)-transformed cells.*

of the gel (0.7 cm). However, in each experiment the positions of the peaks in TK activity from the transformed or lytically infected cells were clearly different from the positions of the peaks of $L_b$ TK activity.

**Thermal inactivation of gel fragments.** Enzyme extracts from $L_b$ cells lytically infected with HSV-1 or with HSV-2 were subjected to polyacrylamide gel electrophoresis. A slice (5 mm) was cut from within the region of the peak in TK activity in each gel. Gel fragments were incubated at 41.5 C for 3 h. The results indicate that the HSV-2 TK was highly thermolabile with respect to the HSV-1 TK (residual activities of 1 and 66%, respectively). The difference in thermostabilities of the HSV-1 and HSV-2 TKs is, therefore, probably not due to the presence of a heat labile cofactor (separable by gel electrophoresis) in the crude enzyme preparations.

## DISCUSSION

$Ltk^-$ cells and HeLa Bu cells are incapable of utilizing exogenous thymidine (9). Ultraviolet-irradiated HSV-1 or HSV-2 can stably confer the ability to utilize exogenous thymidine to either cell type. We have shown previously, by polyacrylamide gel electrophoresis, that a new TK is present in HSV-1-transformed L cells. This new TK is similar to the TK induced during lytic infection by HSV-1 and different from the TK present in normal L cells (12).

A new TK is also present in HSV-2-transformed L cells. The TK present in HSV-2-transformed L cells is highly thermolabile relative to the TK present in HSV-1-transformed L cells or in normal L cells. The TK present in HSV-2-transformed L cells and the TK induced during lytic infection by HSV-2 exhibit similar kinetics of thermal inactivation. The TK from HSV-2-transformed HeLa Bu cells is highly thermolabile relative to the TK present in HSV-1-transformed HeLa Bu cells. The thermolability of the TK from HSV-2-transformed HeLa Bu cells is similar to the thermolability of the TK from HSV-2-transformed L cells. These results suggest that the new TKs present in HSV-1- and HSV-2-transformed cells are distinct from each other. Furthermore, the new TKs present in HSV-1- or HSV-2-transformed human cells are similar to the new TKs present in HSV-1- or HSV-2-transformed murine cells. The properties of the new TKs present in the HSV-transformed cells are, therefore, determined by the type of the virus used for transformation and not by the type of cell being transformed. This result is consistent with the hypothesis that functional viral genes coding for TK have been stably transferred to the HSV-transformed cells.

We have not rigorously excluded the possibility that the new TKs present in the HSV-1- and HSV-2-transformed cells are, in fact, coded for by cellular TK genes which are not normally expressed in tissue culture. Similar unexpressed cellular TK genes could conceivably be present in both mouse and man. At present, in vitro production of TK from purified HSV DNA would be the only conclusive proof that HSV carries a structural gene coding for TK. Furthermore, it would have to be proved that the TK produced in vitro is identical to the TK induced by HSV during lytic infection.

Physical evidence for the stable incorporation of herpes simplex genetic material into mammalian cell genomes has been presented recently by a number of investigators. Frenkel et al. (7) have found RNA which hybridizes to HSV-2 DNA in tissue from human cervical carcinoma, and Royston and Aurelian (15) have found HSV antigens in exfoliated cells from human cervical carcinomas. Duff and Rapp (4, 5) and Kutinova et al. (11) have found HSV-specific antigens in hamster fibroblasts oncogenically transformed in vitro by HSV-2.

In this report, we provide evidence that HSV can stably transfer a functional gene coding for a known enzyme to mammalian cells which lack this enzymatic function. Presently, we are exploring the possibility that HSV may incorporate cellular structural genes at low frequency. If this proves to be so, it may be possible to select for a population of HSV which carries a specific cellular gene; such a virion might then be used to transfer this gene to a recipient cell deficient in this function.

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