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Expression of the Viral Thymidine Kinase Gene in Herpes Simplex Virus-Transformed L Cells

SHIE-SHENG LIN AND WILLIAM MUNYON
Department of Medical Viral Oncology, Roswell Park Memorial Institute, Buffalo, New York 14203

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In these studies, the expression of thymidine kinase (TK) in normal and herpes simplex virus (HSV)-transformed L cells has been compared. In asynchronously dividing cultures of L cells, the TK activity rose and declined rapidly and coordinately with DNA synthesis. When net cell increase stopped, TK activity was at a minimum. In contrast, TK activity of HSV-transformed cells remained at a minimum during rapid DNA synthesis and gradually increased as the rate of DNA synthesis decreased. When net cell increase stopped, TK activity was at a maximum. In synchronous cultures of L cells, TK activity rose and fell coordinately with the rate of DNA synthesis. In synchronous cultures of HSV-transformed cells, no increase in TK activity was observed during the period of rapid DNA synthesis, i.e., the S phase. These findings indicated that the viral TK gene in HSV-transformed cells was not placed under the control of the cellular mechanisms which normally modulate the host cell TK gene. Lytic infection of HSV-transformed cells with a TK^- mutant of HSV-1 induced a four- to fivefold increase in viral TK. The TK of HSV-1 was induced in the HSV-1-transformed cells and HSV-2 in the HSV-2-transformed cells by this TK^- mutant. The same infection of normal L cells decreased the cellular TK activity by 80%. This stimulation, rather than inhibition, suggest that the viral gene in HSV-transformed cells retain some of its original viral characteristics.

Thymidine kinaseless L cells (Ltk^-) have been inheritably transformed to cells having a thymidine kinase (TK) positive phenotype, by nonlytic infection with ultraviolet light-irradiated herpes simplex virus (HSV) (19). Several lines of evidence indicate that this acquired TK-positive phenotype in the transformed cells is due to the presence of a viral TK gene. Thus, the TK from the transformed cells has electrophoretic mobility identical to that of TK present in Ltk^- cells lytically infected with HSV (18). The thermal stability of the TK from HSV type 1 (HSV-1)-transformed L cells is similar to that of TK from Ltk^- cells lytically infected with HSV-1, while the TK from HSV-2-transformed cells (from both mouse L cells and HeLa cells of human origin) is unstable at 41 C as is the TK from HSV-2 lytically infected cells (6). It is reasonable to assume, therefore, that the 10- to 15-fold increase in TK activity in the HSV-transformed L cells results from the expression of the HSV gene coding for TK.

With the HSV-transformed L cells available, it was of interest to determine whether the foreign TK gene, when donated to a mammalian cell, would be placed under normal cellular control; that is, would the activity of this TK gene rise and decline coordinately with cellular DNA synthesis activity?

The rationale of our approach was based on the well-documented information regarding the periodicity of cellular TK expression in cell culture. TK activity of cultured mammalian cells (3, 9, 13-15, 17) and the cells of regenerating liver (23) is finely controlled by some cellular mechanisms (not yet conclusively defined) which permit expression only in the rapid growth stage of the culture and in the early S phase of the cell cycle. Thus, if the viral TK gene acquired in the HSV-transformed cell was under the control of the mechanism which normally controls the cellular TK gene, its expression pattern during synchronous and asynchronous growth should have been similar to that of normal L cells. Furthermore, under environmental influences, they should have responded in the same way as the L cells as far as TK activity is concerned.

As shown later, under various conditions, the pattern of the expression of the TK activity in the HSV-transformed cells was completely different from that of normal L cells. The results suggest that the viral TK gene present in the

HSV-transformed cells was not under control of the cellular mechanisms which govern the cellular TK gene. This difference may have been due to: (i) a structural difference between the viral TK gene and the cellular TK gene; (ii) differently residing site between the viral TK gene and the cellular TK gene; (iii) failure of the viral TK gene to attach to cellular DNA (i.e., its existence as a plasmid); or (iv) a defect in the TK regulatory mechanism of the HSV-transformed cells.

MATERIALS AND METHODS

Cell lines. L cells and HSV-1- and HSV-2-transformed L cells (Cl 139 and Cl 207, respectively) have been described by Munyon et al. (19) and Davis et al. (6).

Viruses. Wild-type HSV-1 and its mutant B2006 (deficient in TK gene [7]) were used.

Media. Medium EM-5CS, composed of Eagle minimal essential medium (8) supplemented with nonessential amino acids and 5% calf serum, was used for routine growth culture of L cells. For routine growth culture of HSV-transformed cells, medium EM-5CS-MTAG was used, composed of EM-5CS containing methotrexate (6×10^{-7} M), thymidine (TdR, 1.6×10^{-5} M), adenosine (5×10^{-5} M), and guanosine (1×10^{-5} M). Medium EM-5CS-TAG, which is the same as EM-5CS-MTAG with the omission of methotrexate, was used for short-term experiment culture of both L and HSV-transformed cells. To measure tritiated thymidine ([^{3}H]TdR) uptake, medium EM-5CS-AG (the same as EM-5CS-TAG except TdR was completely deleted) was used.

Tritiated thymidine ([^{3}H]TdR). The chemical [^{3}H]TdR with specific activity of 20 Ci/mmol was purchased from New England Nuclear (Boston, Mass.).

Cell number measurement. Cell number was counted in an electronic particle counter (electrozone celloscope) of Particle Data, Inc. (Elmhurst, Ill.).

Radioactivity measurement. Radioactivity of all samples was estimated at ambient temperature in a Beckman liquid scintillation counter model LS-230.

Cell culture. In all cases, monolayer culture at 37 C in a humidified atmosphere of 10% carbon dioxide and 90% air was used. Medium used for the culture varied with the experimental condition. In general, medium EM-5CS was used for routine growth of L cells, whereas medium EM-5CS-MTAG was used for HSV-transformed cells in order to prevent the accumulation of TK revertant in the transformed population. When L cells and HSV-transformed cells were compared, medium EM-5CS-TAG was used. Subculture was generally performed with 3- to 4-day-old culture except as otherwise specified. Cells were resuspended by trypsinization.

Lytic infection of cells with HSV. In general, a 24-h monolayer was infected at a multiplicity of 10 PFU/cell for 1 h. Then the virus suspension was aspirated, and infected cells were cultured in medium EM-5CS-TAG. Controls were treated in the same manner except that the virus was omitted.

Separation of cytoplasm, nuclei, and mitochondria. Procedures described by Schneider et al. (21) were used. For comparison of TK activity in these fractions, each fraction was separately sonicated and made up to same volume with the TK sonication buffer as specified below.

Preparation of TK extract. To prepare the extract for TK assay, cells were harvested by trypsinization (about 3 min) and washed twice with Eagle minimal essential medium (8) without phenol red by means of suspension and centrifugation ($800 \times g$) at 4 C, and drained for 5 min at 2 to 4 C in a refrigerator. The cell pellet was then thoroughly suspended in a sonication buffer (0.2 ml/10^6 cells) containing 20% glycerol, 0.001 M dithioerythritol (Sigma Chemical Co., St. Louis, Mo.) and 0.005 M maleate tris(hydroxymethyl)-aminomethane at pH 6.5. The cell suspension was stored at −70 C until the TK assays were done. Control experiments showed that the TK activity (stored as described) was stable for at least 2 weeks. We also found that trypsinization of the monolayer culture did not decrease the TK activity (when compared to the scraping of the monolayer culture). Immediately before TK assay, the cell suspension was thawed and sonically treated in a well-type sonic oscillator model DF101 (Raytheon Manufacturing Co., Lexington, Mass.) for 2 min at maximum strength at 4 C. The sonically treated suspension was centrifuged in a refrigerated Sorvall centrifuge at $30,000 \times g$ for 30 min, and the supernatant was used for TK assay. Under these conditions, only the cytoplasmic TK was present in the extract.

TK assay. TK assay was exclusively carried out with an interface reaction method recently developed in our laboratory. Briefly, the phosphorylation reaction was performed on DEAE paper squares (Whatman DE-81) instead of in the usual liquid phase reaction. Whatman DE-81 paper was cut into 1-inch square pieces and supported on caps of scintillation vials (Wheaton vitro 180, with polyethylene snap cap); the caps were loaded with about 0.1 ml of distilled water and placed in a six-well plastic culture dish (catalog no. FB-6TC, Linbro Chemical Co., Conn.). The cap prevents the reaction mixture from touching the well once it is absorbed to the paper, and the reproducibility of the assay is thus improved. The small amount of water in the cap functioned to keep the DEAE paper moistened so as to facilitate the enzymatic reaction. To perform the assay, a 20-microliter sample (per square) of the TK reaction buffer (composed of maleate tris, 0.01 M at pH 6.5; [^{3}H]thymidine, 2×10^{-5} M with a total radioactivity of 2 μCi; $MgCl_2$, 0.006 M; adenosine triphosphate, 0.02 M; and KCl, 0.2 M) was applied to the center of the paper square, and followed with 20 μliters of TK extract as prepared above. The whole culture dish with the loaded papers was then incubated for 30 min in a humidified carbon dioxide incubator without cover. We noted that the unusual high ratio of [ATP] to [Mg^{2+}] (about 3) in the present method increased the sensitivity of the assay four- to

fivefold as compared to that commonly used with a [ATP] to [Mg^{2+}] ratio of 1 in the liquid-phase reaction, and also extended the linearity of the reaction to a wider range of enzyme concentration (Fig. 1). At the end of 30 min, the reaction was stopped by dipping the DE-81 paper square in a beaker containing about 200 ml (per two paper squares) of 0.003 M ammonium formate buffer (pH 6.0) overnight. The washing buffer was then decanted, and the square was washed twice again with the fresh buffer (about 30 min per wash). Finally, the square was washed briefly with distilled water and placed in a scintillation vial for drying overnight in a 100 C oven. A 10-ml sample of toluene scintillation liquid was added to each vial and counted. A counting efficiency of about 6.7% was estimated by channel-ratio method. If the DEAE squares were completely dried, the efficiency remains very constant. The counting efficiency could be further enhanced to 25 to 30%, if the square was first digested in 1 ml of 0.1 N NaOH for 1 h and counted with an additional 9 ml of toluene scintillation liquid containing 50% Triton X-100. This procedure requires several more steps and therefore was not routinely used. The background count was estimated with duplicate control DEAE squares for which 20 μliters of plain sonication buffer was used instead of TK extract; they were processed in the same way as described. The net counts per minute (count/min) (after correction for background) was then converted to picomoles of TdR phosphorylated per milligram of protein in the TK extract.

Protein determination. Protein was quantitated by the method of Lowry et al. (16) using bovine serum albumin as a standard.

Estimation of DNA synthesis activity. The DNA synthesis activity of the monolayer culture was estimated by measuring the amount of [^{3}H]TdR incorporated into a cold trichloroacetic acid precipitate. The medium in the 6-cm petri dish (Falcon) was aspirated and replaced with 3 ml of warm (about 38 C) medium EM-5 CS-AG containing 2 μCi [^{3}H]TdR per ml with a specific activity of 20 Ci/mmol. The culture was incubated at 37 C for 60 min in a carbon dioxide incubator except when otherwise specified. Then the radioactive medium was aspirated and washed three times with Eagle minimal essential medium (8) solution. The washed monolayer was covered with 3 ml of cold 5% trichloroacetic acid solution and stored at 4 C overnight. The trichloroacetic acid solution was aspirated, and the dishes were washed twice (3 ml of 95% ethanol) and dried. The bottom of the dish, containing the fixed cells, was then cut into small pieces and dissolved in 20 ml of toluene scintillation liquid, and the radioactivity was determined. In this case, the counting efficiency was about 5.8 to 6.0%. The validity of this measurement was evidenced by a very low count encountered in the monolayer dish of Ltk$^-$ cells and by the radioactivity insoluble in 5% trichloroacetic acid solution.

Cell synchronization. Hydroxyurea (HU) was used to obtain synchronized L cells and HSV-transformed cells; HU kills cells actively synthesizing DNA (S cells) but not the G_1, G_2, and metaphase cells as reported by Sinclair (22). A 24-h old monolayer culture of L cells or HSV-transformed cells was treated with medium EM-5CS-TAG containing 0.5 mM HU for 10 h. Then the HU-containing medium was removed by aspiration, and the culture was washed twice with warm medium EM-5CS-TAG. The monolayer was cultured for 5 h in the plain medium EM-5CS-TAG. The same monolayer was once more treated with HU-containing EM-5CS-TAG for 5 h and released for 5 h as before. An additional 5-h HU treatment was conducted to ensure a high density of cells at the G_1/S boundary. At the end of the third HU treatment, the monolayer was washed and trypsinized in medium EM-5CS-TAG with a cell density of about 0.6 $\times$ 10^6/5 ml and plated into 6-cm Falcon petri dishes. At the indicated time points (see Fig. 3 and 4), three sets of duplicate dishes were removed for separate determinations of cell number, TK activity, and [^{3}H]TdR uptake.

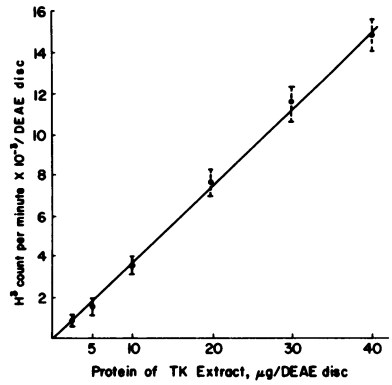

FIG. 1. *TK reaction on DEAE disc with various protein concentration in the TK extract. A TK extract was prepared from a 24-h L cell monolayer culture and diluted to give the indicated protein content per 20 μliters. At each protein level, four TK assays were made with four separate DEAE paper squares. The solid points represent the average TK activity, count per min per square, at each protein level. The horizontal bars below and above the average point refer to the standard deviation of the measurement.*

Electrophoresis. Disc polyacrylamide gel electrophoresis was carried out essentially as described by Munyon et al. (18) with the following modifications: (i) the electrophoresis was conducted at 2 C with 3 mA per gel; and (ii) after electrophoresis, the gel was sliced into 1-mm slices, and each slice was assayed for TK activity by incubating a 1-mm slice for 1 h at 37 C in 0.1 ml of TK reaction buffer (specified before) plus 1 mM dithioerythritol. Some 25-μliter samples of reacted mixture were spotted to DEAE paper, washed, and counted as described proviously.

Thermal stability. The thermal stability experiment was performed by the method of Davis et al. (6).

RESULTS

Factors influencing the TK assay. The TK assay method used in this study was quantita-

tive, as evidenced by the linearity of the reaction over a wide range of the enzyme concentration (Fig. 1). The conditions for the TK assay were the result of numerous control experiments in both normal and HSV-transformed L cells to determine: (i) the optimal washing condition which retained maximum amount of dTMP (thymidines'-monophosphate) and least TdR; (ii) the optimal concentration of ATP, Mg^{2+}, and the optimal ratio between the two; (iii) the optimal condition to store the sample for TK assay with minimum loss in TK activity; (iv) the composition of sonication buffer that gave the highest TK activity; and (v) the kinetics of the TK reaction on the DEAE square. Generally, 99.9% of unreacted TdR could be removed under the conditions described above, and thus a background of about 300 counts/min resulted. A TK extract of 2×10^5 rapidly growing normal L cells gave about 3×10^4 to 4×10^4 counts/min and about 5% error with this method. However, when the protein level of the TK extract was lower than 1 μg per 20 μliters, the error may have been as high as 20%, due to various factors encountered in any quantitative measurement. Therefore, the assay should be carried out with a minimal protein concentration, higher than 2 μg per 20 μliters of the TK extract. This corresponds to a total cell number of 2×10^4 to 3×10^4, a number easily met under ordinary experimental conditions.

In comparison to the conventional method in which the TK reaction proceeds in a homogenous liquid phase, the present interface reaction (as reported in this paper) was slower. A kinetic study of the TK reaction with the same reaction mixture composition showed that the interface reaction was maximized in about 50 min at 37 C, whereas the liquid-phase reaction peaked in about 40 min in a shaking water bath (37 C). Nevertheless, the interface method has the advantage over the liquid-phase method since a smaller sample is required, the radioactive substrate ([^{3}H]TdR) is fully utilized, and smaller errors result because less pipetting is needed.

TK activity and DNA synthesis activity in asynchronous cultures. Since the TK activity in an asynchronized L cell culture has been known to increase coordinately with DNA synthesis activity (9, 13–15), we examined whether TK activity in the HSV-transformed cells follows the same pattern observed in normal L cells. As shown in Fig. 2 and 3, the TK activity of HSV-1-transformed cell Cl 139 showed a pattern completely different from that seen in normal L cells.

In the case of L cells (Fig. 2), TK activity rose sharply along with the rapid DNA synthesis and dropped nearly to zero as the DNA synthesis declined to minimum. The cell number came to a stationary state on 3 days after subculture. This general pattern in which TK activity closely coordinated with DNA synthesis, was repeatedly observed in a number of similar experiments with L cells and was in good agreement with results of others (9, 13–15).

When the asynchronous HSV-transformed cell was used in the same type of experiment (Fig. 3), the coordinated pattern between TK and DNA synthesis was not found. Instead, the TK activity slightly declined or remained at the same level, although DNA synthesis activity increased to peak value 1 day after subculture. When the DNA synthesis activity sharply declined 2 days after subculture, the TK activity rose continuously. Thus, the TK activity in HSV-transformed cells was highest when cell growth stopped and the cultures became confluent. This absence of coordination between TK activity and DNA synthesis was also observed

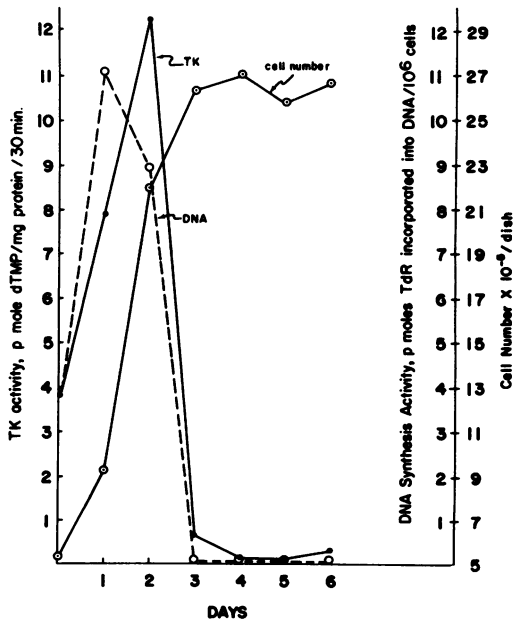

FIG. 2. *Chronological TK activity, DNA synthesis activity, and cell growth (changes of cell number) in a long-term asynchronized L cell culture. L cell suspension was prepared from a 4-day-old culture in EM-5CS-TAG medium and plated into 6-cm Falcon plastic petri dishes (about 0.5 × 10^6 cells each). At indicated time intervals, separate duplicate dishes were removed to measure TK activity, DNA synthesis activity, and cell number.*

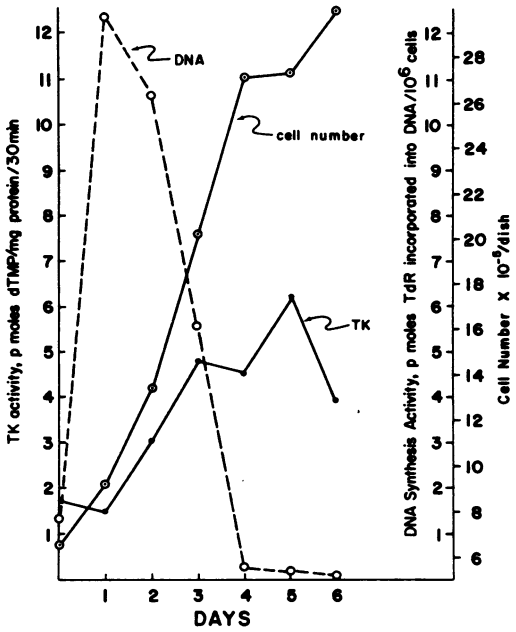

FIG. 3. *Chronological TK activity, DNA synthesis activity, and cell growth in a long-term, asynchronized, HSV-1-transformed cell (Cl 139). Procedures are the same as described in legend to Fig. 2.*

in experiments with HSV-2-transformed cells, such as Cl 207.

TK activity and DNA synthesis in synchronized L cells. Having observed the noncoordinated TK activity with the DNA synthesis activity in the HSV-transformed cells with asynchronous culture, we then analyzed the S phase of an HSV-transformed L cell culture for a possible TK peak. Thus, L cells and HSV-2-transformed cell Cl 207 were synchronized with repeated HU treatment. Samples were removed at different time intervals (Fig. 4 and 5) for the determination of TK activity, DNA synthesis activity, and cell number. Indeed, in the case of synchronized L cells, when the cells were released from the G_1/S boundary and started to enter S phase, a burst of TK activity appeared at 30 min (Fig. 4). This was accompanied by a sharp rise of DNA synthesis which peaked about 3 h later. Both TK activity and DNA synthesis then declined toward the end of S phase. The TK and DNA synthesis activity rose in a parallel manner again in the second S phase of the experiment. In contrast as in the case of the HSV-2 transformed cell Cl 207, the TK activity remained very low during the whole S phase and during all other phases of the cell cycle, in spite of the sharply rising DNA synthetic activity after cells were released from the arrest at the G_1/S boundary (Fig. 5). Therefore, TK activity and DNA synthesis activity were well coordinated in synchronized L cells, but not in synchronized HSV-transformed cells. This was consistent with the result obtained in the asynchronous cultures.

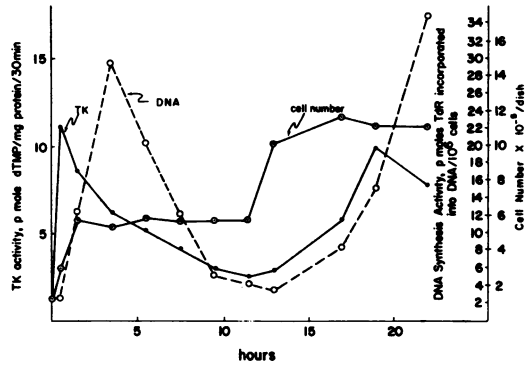

FIG. 4. *TK activity and DNA synthesis activity in the cell cycle of a HU effected, synchronized L cell culture. Synchronized L cells were obtained by three-cycle HU treatment. A cell suspension was prepared in EM-5CS-TAG medium and plated into 6-cm plastic petri dishes for culture. At indicated time points, separate duplicate dishes were removed to measure TK activity, DNA synthesis activity, and cell number. Note that the increase in cell number between period of 0 and 1.0 h does not indicate mitosis, because cells did not completely attach to the petri dishes in this period. All measurements of TK activity, DNA synthesis activity, and cell number in this study were based on attached cells.*

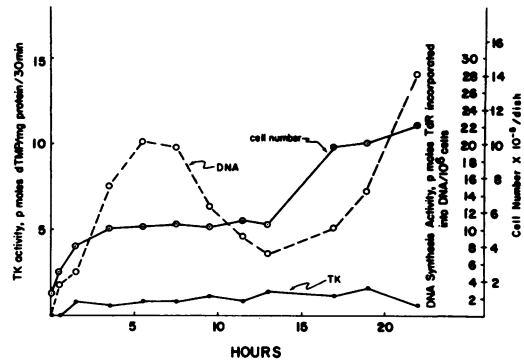

FIG. 5. *TK activity and DNA synthesis activity in the cell cycle of HU-effected synchronized HSV-2 transformed L cells (Cl 207). Procedures are the same as described in legend to Fig. 4. Note that the increase in cell number between period of 0 to 1.5 h does not indicate mitosis, because the cells did not completely attach to the petri dishes during this period. All measurements of TK activity, DNA synthesis activity, and cell number in this study were based on attached cells.*

The cell population used in these experiments was highly synchronized after 3 cycles of HU treatment, as shown by the nearly two-fold increase in cell number at the end of metaphase and a burst appearance of DNA synthesis activity after the cells were released from G_1 arrest. As a control, a separate experiment was carried out in the same manner except that HU was omitted from the medium. In these experiments, no DNA peaks were observed in the time interval 4 to 5 h after release from G_1 arrest. Instead, the DNA peak appeared between 19 and 22 h.

In the case of synchronized L cells, the TK peak preceded the DNA peak by about 3 h. This was in good agreement with the results reported by Smellie (23) who found in regenerating liver (a naturally synchronized cell population) that the initial increase in TK activity precedes DNA synthesis by about 14 h and then it continues to rise with the increase of DNA synthesis, reaching maximum activity about 10 h before the peak of DNA synthesis. In fact, in every one of our five experiments with L cells synchronized with one to three cycles of HU treatment, the TK peak always preceded the DNA peak. However, our results in this context were slightly different from those of others in HeLa cells (3) and L cells (17). These investigators found that the DNA peak either precedes or coincides with the TK peak. The source of such a discrepancy is not clear at the present time. As a whole, however, a coordinated pattern between TK activity and DNA synthesis activity in L cells was clearly shown in all cases. This natural pattern was clearly absent in HSV-transformed cells which carry the viral TK gene, further suggesting that the viral TK gene in the HSV-transformed cell is not under control of the host cell.

Intracellular distribution of the TK activity in L cells and HSV-transformed cells. Experiments with both synchronized and asynchronous cultures strongly suggest that the viral TK gene in the HSV-transformed cells is not under control by the same cellular mechanism which controls the host cell TK gene. Additionally, substantial data have been accumulated to indicate that the TK enzymes in normal L cells and HSV-transformed L cells are different. We, therefore, determined whether these different TK enzymes were present in the same or different cell compartments. Accordingly, stationary and rapidly dividing cultures of both L cells and HSV-transformed cells were processed to separate cytoplasmic, nuclear, and mitochondrial fractions as Schneider and Kuff (21). Table 1 shows the intracellular distribution of TK activity in these cells.

It appears that the TK activities of both normal and HSV-transformed L cells were predominantly found in the cytoplasms. When compared with cultures of transformed cells (23 to 24 days old) and normal L cells, an increased percentage of TK activity was found in the mitochondrial fraction of the transformed cells at 24 h. No further studies have yet been made on this observation. It is of interest that the 23- and 24-day-old cultures of transformed cells had much greater TK activity than the 1-day-old culture (Table 1).

Effect of lytic infection with thymidine kinaseless HSV mutant on the expression of the viral TK gene in HSV-transformed cells. The foregoing data consistently indicate that the viral TK gene present in the HSV-transformed cell is expressed independently and is not subject to the control by the same mechanism which modulate the cellular TK gene; i.e., the viral gene is expressed inde-

TABLE 1. *Intracellular distribution of TK activity in L cells and HSV-transformed cells*[a]

Cell lines	Age of culture	Cytoplasm	Nuclei	Mitochondria
L cells	17 days[b]	4,699 (91)	250 (5)	202 (4)
	24 h	41,630 (96)	926 (2)	969 (2)
Cl 139	23 days[b]	9,785 (98.5)	45 (0.5)	95 (1)
	24 h	4,100 (84.5)	30 (0.6)	709 (15)
Cl 207	24 days[b]	39,405 (97)	468 (1)	558 (1)
	24 h	8,160 (89.6)	82 (1)	864 (9.4)

[a] Cytoplasmic, nuclear, and mitochondrial fractions were separated from 1.5×10^7 cells by the method of Schneider and Kuff (21). Each fraction was separately sonicated and brought up to a total volume of 1 ml of TK extract, and a 20-μliter sample of each was assayed for TK activity. Data are the average of two samples in duplicate and are expressed as counts per min per 20 μliters of respective fraction. Figures in parenthesis refer to the percentage of the TK activity in the indicated fraction of the total.

[b] These cells were incubated at 37 C in the medium EM-5CS-TAG for the indicated number of days without a medium change.

pendently. It is possible that the TK gene of transformed cells still retains a sensitivity to virus-induced controls. Dubbs and Kit (7) have shown that an HSV mutant (B2006), which does not induce TK activity during lytic infection, caused a decrease in activity of cellular TK activity after infection. Therefore, we have carried out experiments to determine the effect of the mutant B2006 infection on the TK activity of HSV-transformed cells. The results are summarized in Table 2. The results of one kinetic experiment on the infection of normal L cells and HSV-transformed cells Cl 207 with this HSV mutant are shown in Fig. 6.

As shown in Table 2, the TK activity of HSV-transformed cells lytically infected with HSV TK$^-$ mutant B2006 (7) was enhanced after 24 h by about 430%, while the same infection of L cells inhibited TK activity by 80%. This not only reflects the difference of the viral TK gene in the transformed cell from the cellular TK gene in the L cells, but also suggests that the viral TK gene retains its viral characteristics. As a result, the lytic infection with mutant B2006 did not suppress its expression, but rather triggered a strong enhancement of the viral TK activity.

To test the nature of the stimulation of TK

TABLE 2. *Effect of 24-h lytic infection of L cells and HSV-transformed cells with wild-type HSV and HSV TK$^-$ mutant (HSV-B2006) on TK activity*

Infection type	L cells	Cl 139	Cl 207
Control	1,211[a]	208	123
Infected with HSV mutant B2006	292	901	537
	(24%)[b]	(433%)	(437%)
Control	1,200	292	232
Infected with wild-type HSV	4,200	3,028	2,816
	(350%)	(1,037%)	(1,200%)

[a] Counts per minute per microgram of protein.
[b] Infected/control.

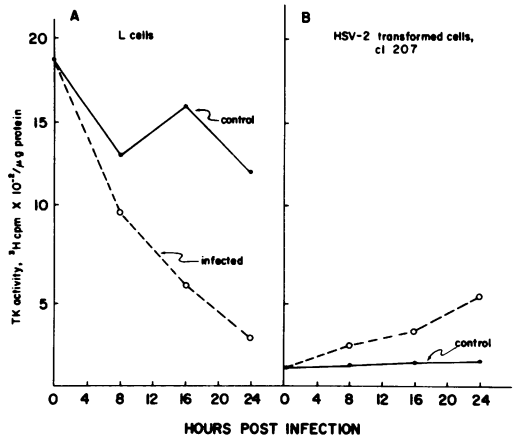

FIG. 6. *Effect of HSV mutant B2006 (TK$^-$) lytic infection on TK activity of L cells (A) and Cl 207 (B).*

activity in HSV-transformed cells and the inhibition of TK activity in L cells by the mutant B2006 infection, a mixed-extract experiment was carried out; a TK extract from infected cells was mixed with one from the noninfected (control) cells. Before TK assay, all extracts were incubated for 30 min at 37 C. The results are summarized in Table 3. It appeared that both TK extracts from the HSV TK$^-$ mutant-infected L cells and infected HSV-transformed cells (Cl 139) contained factors inhibitory to the TK activity at the enzyme level. The four- to five-fold stimulation of TK activity in the HSV-transformed cell by the HSV TK$^-$ mutant infection was apparently not caused by the presence of TK activator or by molecular complementation between the infected and uninfected cell.

To further understand the type of TK induced in the transformed cells by the HSV-1 mutant B2006, polyacrylamide gel electrophoresis was conducted. It has been shown (6, 18) that both HSV TKs have a higher electrophoretic mobility (R_f) than the cellular cytosol TK (predominant). As shown in Table 4, the pre-

TABLE 3. *TK activity of mixed extracts from HSV TK$^-$ mutant-infected cells and uninfected cells*

TK extract (10 μliters used for assay)	TK activity[a]	If no interaction[a]	Effect of mixing on TK activity
Control L cells	41,512		
HSV TK$^-$-infected L cells	1,124		
Mixed extract of above two (10 μliters each)	21,985	42,636	50% Inhibition
Control, Cl 139	678		
HSV TK$^-$-infected Cl 139	2,491		
Mixed extract of above two (10 μliters each)	2,136	3,169	30% Inhibition

[a] Counts per minute of [^{3}H]dTMP.

dominant TK (about 90%) in HSV-transformed cells (both Cl 139 and Cl 207) induced by infection of HSV mutant B2006 has an R_f value of 0.36 to 0.39, comparable to that of TK from HSV-1- and HSV-2-infected Ltk$^-$ cells and HSV-transformed cells. These results together with those shown in Table 2 suggest that the mutant B2006 specifically induced HSV TK but not cellular TK.

Since HSV-1 TK and HSV-2 TK are not distinguishable on the basis of electrophoretic mobility, we performed thermal stability experiments by the method of Davis et al. (6). The results are summarized in Table 5. It is clear that the viral TK induced in the HSV-1-transformed cells (Cl 139) by the mutant B2006 was similar to that of HSV-1-infected Ltk$^-$ cells in thermal stability. Both are more thermally resistant than the HSV-2 TK. On the other hand, the TK present in the B2006-infected Cl 207 was similarly thermolabile to that in the HSV-2-infected Ltk$^-$ cells. All these observations suggest that this HSV-1 mutant B2006 induces HSV-1 TK in HSV-1-transformed cells and HSV-2 TK in HSV-2-transformed cells, dependent on the type of HSV TK gene present in the cells.

DISCUSSION

In this study, we have shown that the TK activity in the HSV-transformed cell, in contrast to the TK activity in normal L cells, was

TABLE 4. *Relative electrophoretic mobility (R_f) of TK in various cell extracts*

Source of cell extract[a]	R_f of TK peaks		
	1	2	3
Normal L cells	0.22 (1.05×10^6; 96)[b]		0.78 (4.2×10^3; 4)
HSV-1-infected Ltk$^-$ cells		0.38 (5.4×10^6; 100)	
HSV-2-infected Ltk$^-$ cells		0.37 (4.8×10^6; 100)	
HSV-1-transformed Ltk$^-$ cells (Cl 139)		0.37 (2.8×10^4; 96)	0.65 (1.2×10^3; 4)
HSV-2-transformed Ltk$^-$ cells (Cl 207)		0.38 (3.5×10^4; 94)	0.65 (2.1×10^3; 6)
B2006-infected Cl 139		0.39 (6.3×10^6; 98)	0.68 (1.1×10^4; 2)
B2006-infected Cl 207		0.36 (3.0×10^6; 97)	0.65 (9×10^3; 3)

[a] All virus infections were effected with 20 PFU/cell for 16 h. The amount of protein loaded to a gel varied from 100 μg to 150 μg in a total volume of 50 μliters.

[b] The figures in the parentheses indicate, respectively, the total counts of [^{3}H]dTMP per minute in the peak fractions and the percentage of counts of [^{3}H]dTMP per minute in the specific peak among all detectable peaks.

TABLE 5. *Thermal stability of TK from extracts of HSV-infected Ltk$^-$ cells and HSV mutant B2006-infected HSV-transformed cells*[a]

Cell type	Residual TK activity at time intervals (min)				
	0	30	60	120	180
HSV-1-infected Ltk$^-$	58,928[b] (100)[c]	29,649 (50.3)	19,858 (33.7)	9,016 (15.3)	3,594 (6.1)
HSV-2-infected Ltk$^-$	47,795 (100)	9,883 (20.1)	3,213 (6.7)	1,632 (3.4)	883 (1.8)
B2006-infected Cl 139	67,908 (100)	32,256 (47.5)	27,163 (40)	15,459 (22.9)	7,152 (10.6)
B2006-infected Cl 207	61,823 (100)	5,441 (8.8)	530 (0.85)	118 (0.2)	115 (0.2)

[a] Total amount of protein of all TK extracts was adjusted to 2 mg of protein per ml for the thermal inactivation.

[b] Counts of [^{3}H]dTMP per 20 μliters of extract per minute, after thermal inactivation (41.5 C) at indicated time intervals (minutes).

[c] Percent of counts per minute at zero time.

not coordinately expressed with the DNA synthesis activity. This was demonstrated in both asynchronized and synchronized culture. Furthermore, lytic infection with a HSV mutant B2006, which does not induce TK during lytic infection, triggered a four- to fivefold increase in TK activity in the HSV-transformed cells, while it suppressed by 80% the TK activity in normal L cells. These data strongly suggest that the viral TK gene in the HSV-transformed cell is not controlled by the same mechanism(s) which governs the cellular TK gene. The failure of the cell to control the viral TK gene may be attributed to many factors. The viral TK gene may be structurally different from cellular TK gene, as shown from the difference in properties of two TKs (6, 18). The viral TK gene may not necessarily be integrated into the same site where the normally cellular TK gene resides, and thus might fail to be controlled by the cellular promoter and/or regulator gene, assuming the Jacob-Monod model of protein regulation is tenable in mammalian cells.

Alternatively, the incoordination between the TK synthesis and DNA synthesis in the HSV-transformed cells may result from a defective regulatory mechanism in the transformed cells effected by the selection medium or by infection of the cells with UV-irradiated HSV. Precise assignment of these interpretations awaits further studies.

The stimulation of the viral TK activity of the HSV-transformed cells by the HSV-mutant B2006 infection implies that some or all of the viral characteristics of the viral TK gene are retained through the transcription and translation steps. It has been shown that most cellular protein synthesis is inhibited subsequent to HSV infection (10, 11). The ability of HSV-mutant B2006 infection to stimulate the viral TK activity may therefore indicate that the TK gene in the HSV-transformed cells is partially suppressed, and that the B2006 infection is able to derepress its activity. Since the stimulation of TK activity of the HSV-transformed cells was neither due to the activation of TK enzyme nor due to the molecular complementation between the TK fragments from B2006-infected cells and the uninfected cells (Table 3), it is likely that the stimulation results from specific facilitation of TK gene replication, transcription, or translation mediated by the HSV-induced factor(s). HSV-specific DNA polymerase (12), RNA polymerase, and derepressor proteins are some possible candidates. In this connection, it is interesting to mention the observations reported by Davidson et al. (5), who have shown that their HSV-transformed cells (equivalent to ours) are able to return to a TK-minus phenotype by a process not due to loss of the viral TK gene.

Another implication of our findings is on the possible molecular mechanism of the oncogenicity of herpesvirus (1, 2, 20). The concentration of dTTP and the activity of the DNA polymerase are two important elements in the control mechanism involved in the initiation and termination of DNA synthesis in the S phase of the cell cycle. Herpesvirus has also been known to be able to induce a virus-specific DNA polymerase activity (12). Thus, if a cell is nonlytically infected by a defective herpesvirus which retains its TK gene and a gene which codes for the DNA polymerase, it is possible that the function of these genes could cause such infected cells to acquire malignant growth properties.

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