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DEOXYTHYMIDINE KINASES IN VARICELLA-ZOSTER VIRUS INFECTED AND BIOCHEMICALLY TRANSFORMED CELLS

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SUMMARY Deoxythymidine kinase (TK) activity induced in varicella-zoster virus (VZV)-infected human embryonic fibroblast (HEF) cells was immunologically distinguishable from that in non infected HEF cells and also from that in herpes simplex virus type 1 (HSV-1) infected HEF cells. The TKs in VZV-biochemically transformed cells were immunologically the same as that induced in VZV-infected human cells and immunologically different from that in Ltk$^-$ cells or in HSV-biochemically transformed cells.

One peak of TK activity with an Rm value of 0.8–0.9, corresponding to mitochondrial TK, was observed on polyacrylamide gel electrophoresis of Ltk$^-$ cell extracts. VZV infected Ltk$^-$ cells had two peaks of TK activity with Rm values of 0.45–0.5 (peak I) and 0.8–0.9 (peak II). Peak I and II were concluded to be virus-specific TK and mitochondrial TK, respectively. VZV-biochemically transformed cells had a peak of activity with an Rm value of 0.4–0.5, corresponding to peak I in VZV-infected Ltk$^-$ cells; that is VZV-specific TK activity.

The present study indicates that VZV has a gene coding for its own TK.

INTRODUCTION

Various enzymes related to DNA synthesis have been reported to increase in cells infected with herpes viruses (Kit et al., 1974). In particular, the deoxythymidine kinase (TK) induced by herpes simplex virus (HSV) has been extensively studied and has been used as a marker of expression of the viral genome (Munyon et al., 1972; Ogino et al., 1974; Garfinkel and McAuslan, 1974; Honess and Watson, 1977). Munyon et al. (1971) first demonstrated that cells lacking TK could be stably transformed to the TK positive phenotype after infection of UV-irradiated HSV, and later the fragment of HSV DNA containing the coding region for viral TK was shown to convert the TK-deficient cells into the TK-positive phenotype (Wigler et al., 1977; Maitland and McDougal, 1977; Colbere-Gorapin et al., 1979). Equine herpesvirus typs 1 (EHV-1), another member of the herpesvirus

group, was shown to induce virus specific TK in cells infected with it and to transform TK-negative mouse cells into cells of the TK-positive phenotype (Allen et al., 1978; McGowan et al., 1979). These studies indicated the usefulness of TK in studies on herpesviruses.

Varicella-zoster virus (VZV) has also been reported to induce TK activity in cells infected with it by several investigators including us (Dobersen et al., 1976; Ogino et al., 1977; Hackstadt and Mallavia, 1978; Cheng et al., 1979). Though in these studies the induced TK was shown to differ in some biochemical and biophysical properties from the cellular one, it still remains to be determined whether VZV actually has the capacity to code for its own TK. Recently, we succeeded in converting TK-deficient mouse cells (Ltk^-) to the TK-positive phenotype (Ltk^+) by infecting them with cell-associated VZV and we call these converted cells VZV-biochemically transformed cells by analogy with nomenclature in the HSV or EHV-1 system (Yamanishi et al., 1981).

In this study, we examined whether TK induced by VZV infection was serologically different from cellular TK or HSV-infected TK. We also examined serologically whether the TK present in biochemically transformed cells was the same as that in VZV-infected cells. In addition, we examined the electrophoretic patterns of TKs from VZV-infected and biochemically transformed cells.

MATERIALS AND METHODS

1. *Cells*

Human embryonic fibroblast (HEF) cells and human embryonic lung (HEL) cells were grown as monolayers in 250 ml bottles in medium 199 with 10% fetal calf serum plus $NaHCO_3$ (0.075%), penicillin (100 μg/ml) and streptomycin (100 μg/ml). Ltk^- mouse cells, a mutant cell line resistant to BuDR and deficient in cytosol TK activity (Murayama and Okada, 1970), were also grown in the same medium supplemented with 50 μg/ml of BuDR. The BuDR was omitted from the medium in the cell passage preceeding experiments. L(K) clone 1 and L(O) clone 3, which were biochemically transformed by VZV (Yamanishi et al., 1981), were grown in selective medium (growth medium containing 6×10^{-7} M aminopterin, 5×10^{-5} M guanosine, 5×10^{-5} M adenosine, 10^{-4} M glycine, 5×10^{-3} M glutamine, and 1.6×10^{-5} M of thymidine) and the medium were changed to non-selective medium in the cell passage preceeding experiments.

2. *Viruses*

The Kawaguchi, Oka and Izawa strains of VZV, originally isolated by M. Takahashi in our laboratory, were used. The Seibert strain of HSV (Ogino et al., 1973) was also employed for experiments. Virus preparation and infection with cell-free virus were described previously (Ogino et al., 1973, 1977).

3. *Biochemical transformation*

Biochemical transformation with UV-irradiated HSV was done exactly as reported by Garfinkle and McAuslan (1974). One of the clones, named L (HSV)3–1, was used in this experiment. Biochemical transformation with VZV was performed by infecting Ltk^- cells with VZV-infected HEF cells (Yamanishi et al., 1981). Briefly, sparse monolayers of Ltk^- cells (5×10^5 cells in 100 mm plastic petri dishes) were inoculated with the same amount of VZV-infected HEF cells suspended in 15 ml of growth medium. After 48 h, the medium was changed to the selective medium described above and culture was continued in selective medium. Two transformed clones were used in this experiment. The L(K) clone 1 was obtained by using the Kawaguchi strain of VZV, and the L(O) clone 3 was obtained by using the Oka strain of VZV.

4. *Preparation of cell extract*

Cell extract was prepared as described previously (Ogino et al., 1973, 1977). Briefly, cells ($5–10\times10^6$) were washed three times with phosphate buffered saline (PBS), scraped loose with a rubber policeman, and centrifuged at low speed. They are then suspended in 0.5–1.0 ml of 0.15 *M* KCl in 0.05 *M* Tris buffer (pH 8.0) containing 3 m*M* 2-mercaptoethanol. The suspension were subjected to ultrasonic disintegration, and the sonicated preparation was centrifuged at 20,000 rpm for 60 min in a No. 40 rotor of a Hitachi 55 P centrifuge. The supernatant fractions were used as sources of enzyme extracts. Enzyme extracts were frozen and stored at

−70 C until assayed. Protein in cell extracts was measured by the method of Lowry et al. (1951).

5. *Assay of thymidine kinase activity*

The enzyme activity of the cellular extract was assayed as previously described (Ogino et al., 1973, 1977). The enzyme assay mixtures contained 0.2 μCi of ^{14}C-TdR (56.6 mCi/mmole, Amersham), 5 m*M* ATP, 5 m*M* $MgCl_2$, cell extract and 0.05 *M* Tris buffer (pH 8.0) to give a volume of 0.25 ml. The reaction was conducted at 38 C for 15 min and terminated by immersing the mixtures in a boiling water bath for 2 min. The amount of phosphorylated ^{14}C-TdR was determined by the DEAE-cellulose disc method. Under the conditions used, enzyme activity was linearly related to both the amount of cellular protein and the time of incubation. The specific activity of the enzyme is defined as nanomoles of ^{14}C-TdR nucleotide produced in the reaction per 15 min per milligram of protein of the cellular extract.

6. *Antiserum to thymidine kinase*

Antiserum against VZV-induced TK was prepared in a green monkey by injecting VZV-infected African green monkey kidney cell extracts with Freund's complete adjuvant (Yamanishi et al., 1980). The antiserum against HSV-induced TK was perpared in a New Zealand White rabbit by injecting the semipurified HSV-induced TK with Freund's complete adjuvant. The crude cell extract of HSV-infected New Zealand white rabbit kidney cells was prepared as described for the preparation of cell extract in the Materials and Methods. Crude cell extract was precipitated with ammonium sulfate and then passed through a Sephadex G 100 superfine column (Ogino et al., 1973). The fraction containing TK activity was used as semipurified HSV-induced TK. No cross-reaction between VZV and HSV was observed in a test of neutralization of infectivity. Before use, preimmune and immune sera were exhaustively dialyzed against 0.05 *M* Tris buffer (pH 8.0) and inactivated at 56 C for 30 min.

7. *Neutralization of thymidine kinase activity by antiserum*

A sample of 100 μl of enzyme extract was mixed with 50 μl of anti-serum and incubated for 2 h at 4 C. The mixture was then assay for TK activity as described above.

8. *Acrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis was carried out as described by Gabriel (1971) in 5% acrylamide gel. The running buffer consisted of final concentrations of 24 m*M* Tris and 0.192 *M* glycine at a final pH of 8.3 at 25 C. Electrophoresis was performed for about 2 h at 4 C at a constant current 3 mA/gel. Bromphenol blue was used as a stacking dye. After electrophoresis, the gels were sliced into 3 mm sections, placed in 0.25 ml of the TK assay reaction mixture described above and incubated at 38 C for 120 min. Mobilities were calculated relative to the bromphenol blue dye front.

To see the effect of antiserum on TK activity, the gels were sliced into 5 mm sections and halves of each slice were preincubated for 30 min at 4 C in 150 μl of diluted preimmune and immune serum (one-third dilution with 0.05 *M* Tris buffer, pH 8.0) and then assayed for TK activity as described above.

RESULTS

1. *Neutralization of TK activity from virus infected and non-infected HEF cells with antiserum*

TK activity was induced in HEF cells after infection with VZV as we reported previously (Ogino et al., 1977). The VZV-induced TK activity was efficiently (91.9%) neutralized with anti-VZV monkey serum, but not with preimmune serum. Neither preimmune serum nor immune serum neutralized with TK activity of uninfected HEF cells (Table 1). Similar results were obtained when other strains of VZV (strains Oka and Izawa) were used instead of strain Kawaguchi, or when HEL cells were used instead of HEF cells (data not shown). These results show that VZV-induced TK was immunologically distinct from cellular TK.

To determine whether VZV-induced TK was serologically distinguishable from HSV-induced TK, we tested the inhibitory effect of anti-VZV serum on HSV-induced TK activity. As shown in Table 1, neither anti-VZV serum (preimmune and immune) had any inhibitory effect on HSV-induced TK activity. In ad-

TABLE 1. *Neutralizations of VZV- and HSV-induced TK activity with anti-VZV and anti-HSV serum*[a]

Enzyme extract	TK Activity (% of Control)				
	Control (Tris buffer)	Anti-VZV serum		Anti-HSV serum	
		Preserum	Immuneserum	Preserum	Immuneserum
Uninfected (2.1)[b]	100	95.2	107	109	95.3
VZV-infected (28.9)[b]	100	117	8.1	108	125
HSV-infected (29.2)[b]	100	113	105	102	40.6

[a] The cells used were human embryonic fibroblast (HEF) cells. The viruses used were strain Kawaguchi (VZV) and strain Seibert (HSV). TK activity was assayed as described in the Materials and Methods, and is shown as a percentage of the control (activity in the presence of Tris buffer instead of serum).

[b] Figures in parentheses are specific activities of TK of enzyme extracts.

TABLE 2. *Neutralization of TK activities from biochemically transformed mouse cells by antisera*[a]

Enzyme extract	TK Activity (% of Control)				
	Tris buffer (control)	Anti-VZV serum		Anti-HSV serum	
		Preserum	Immuneserum	Preserum	Immuneserum
L(K) clone 1 (2.4)[b]	100	95.4	1.8	106	107
L(O) clone 3 (2.5)[b]	100	95.0	5.3	110	110
L(HSV) 3-1 (1.2)[b]	100	88.8	91.7	94.9	21.3

[a] TK activity was assayed as described in the Materials and Methods, and is shown as a percentage of the control activity (in the presence of Tris buffer instead of serum). L(K) clone 1 and L(O) clone 3 are mouse cells biochemically transformed by VZV, and L(HSV) 3-1 cells are mouse cells biochemically transformed by HSV.

[b] Figures in parentheses are specific activities of TK of extracts.

dition, anti-VZV rabbit serum, which neutralized HSV-induced TK activity efficiently, did not neutralize the activity of VZV-induced TK. These results show that the VZV-induced TK was also immunologically distinct from the HSV-induced TK.

2. *Neutralization of TK activity from biochemically transformed mouse cells by antiserum*

Ltk$^-$ cells can't survive in selective medium because they have no cytosolic TK, although they have weak mitochondrial TK activity. When Ltk$^-$ cells were infected with VZV-infected-HEF cells, they were converted to the TK-positive phenotype; that is, they could grow in selective medium. As we suspected that they had VZV-specific TK, we tested them for the presence of VZV-specific TK using to immune serum described above.

As shown in Table 2, the TK activity in VZV-biochemically transformed cells L(O) clone 3 was efficiently neutralized with anti-VZV serum. On the other hand, the TK activity in HSV biochemically transformed cells, L(HSV) 3–1, was not neutralized by anti-VZV serum. Anti-HSV serum did not neutralized the VZV-induced TK activity, but it did neutralize the HSV-induced TK activity.

These results showed that the TK activity in VZV-biochemically transformed cells differed immunologically from that of Ltk$^-$ cells but not from that induced in VZV-infected

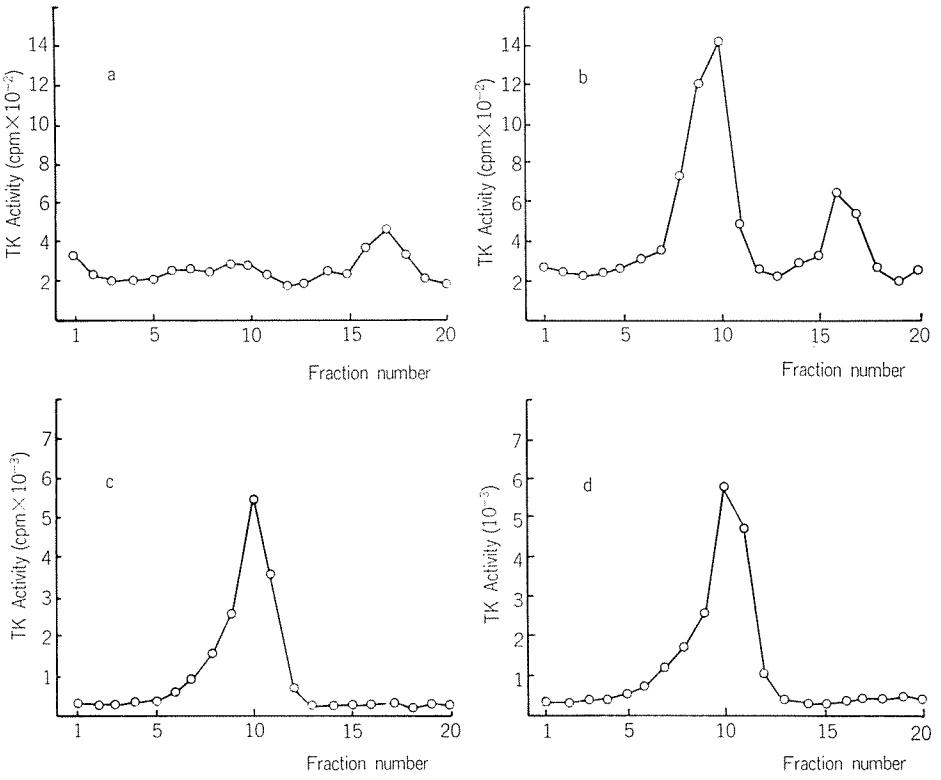

FIGURE 1. Electrophoresis was carried out in 5% acrylamide gel in 8 cm glass columns at 4 C for about 120 min at a constant current of 3 mA per gel. Electrophoresis was stopped when the bromophenol blue band reached the anode of the tube. Then the gel was sliced into 3 mm thick sections in the cold. TK activity was assayed by incubating each slice in 250 μl of reaction mixture (0.2 μCi of ^{14}C-TdR and 5 m*M* $MgCl_2$ with 0.05 *M* Tris, pH 8.0 up to 0.25 ml) at 38 C for 120 min. The reaction products were separated on a DEAE-cellulose paper disc. Mobilities were calculated relative to the bromophonol blue dye front. (a) non-infected Ltk$^-$ cells (b) VZV (strain Kawaguchi) infected Ltk$^-$ cells, 18 h after infection (c) L(K)cl 1 cells (d) L(O)cl 3 cells.

cells, and that it was immunologically different from that of HSV-biochemically transformed cells.

3. *Electrophoretic analysis of TK activity*

The relative mobility (Rm) value of TK activity in Ltk$^-$ cells was found to be 0.85 (Fig. 1a). In other experiments, this value varied from 0.8 to 0.9. This activity was suspected to be due to mitochondrial TK (Munyon et al., 1972; Kit et al., 1973; 1974). When the subcellular distribution of TK activity in Ltk$^-$ cells was examined, most of the activity was found in the mitochondrial fraction and the electrophoretic pattern of TK activity in this fraction was the same as that shown in Fig. 1a (data not shown). From all these results, it was concluded that the peak in Ltk$^-$ cell extracts was mitochondrial TK activity. In contrast, two peaks of activity with Rm values of 0.5 (peak I, range 0.4 to 0.5) and 0.8 (peak II, range 0.8 to 0.9) were found in the electrophoretic pattern of VZV-infected Ltk$^-$ cells (Fig. 1b). No peak corresponding to peak I

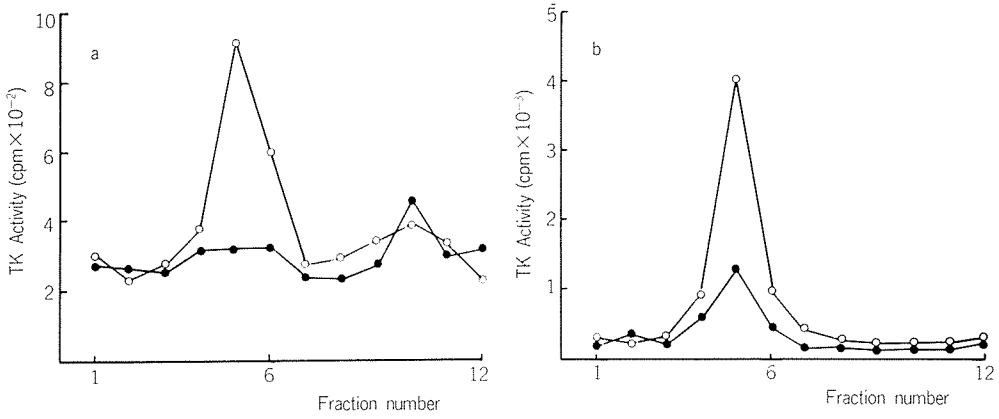

FIGURE 2. Electrophoresis was done as for Fig. 1. After electrophoresis, gels were sliced into 5 mm thick sections. Each section was divided in half and assayed for TK activity by incubation in 250 μl of reaction mixture at 38 C for 120 min after preincubation for 30 min with perimmune (○—○) or immune serum (●—●). Immune serum was perpared in a green monkey by injection of VZV-infected green monkey cell extracts with Freund's complete adjuvant. Before use, sera was exhaustively dialyzed against 0.05 *M* Tris buffer (pH 8.0) and inactivated at 56 C for 30 min. The reaction products were separated on DEAE-cellulose paper paper discs. (a) VZV (Kawaguchi) infected Ltk$^-$ cells (b) L(K)cl 1 cells.

was detected in uninfected Ltk$^-$ cells and peak I disappeared when sliced gels were preincubated with anti-VZV serum, as shown in Fig. 2a. The peak corresponding to peak I was also observed in VZV-infected HEF cells (data not shown). From these results, it was concluded that peaks I and II represent the activities of VZV-specific and mitochondrial TK, respectively. The electrophoretic patterns of TK activities in two biochemically transformed cells lines, L(K) clone 1 and L(O) clone 3, are shown in Fig. 1c and d. Both of these cell lines had a peak of TK activity with an Rm value of 0.5 (range, 0.4–0.5). The location of TK activity in VZV-transformed cells was the same as that of peak I of VZV-infected Ltk$^-$ cells. Although no peak in the position of peak II is seen in Fig. 2b, the activity could be detected on longer incubation during enzyme assay, indicating the presence of a low level of mitochondrial TK (data not shown). When halves of sliced gels were preincubated with anti-VZV serum before enzyme assay, the activity was significantly reduced (to less than one-third of that in the presence of preserum), as shown in Fig. 2b. These results indicate that the peak of activity in VZV-biochemically transformed cells was due to the expression of VZV-specific TK.

DISCUSSION

VZV has been shown to induce TK activity on its lytic infection of human fibroblast cells. The induced TK differed from cellular TK in several biochemical and biophysical properties, including heat-sensitivity, substrate specificity, optimal pH, inhibition by dTTP and electrophoretic mobility (Dobersen et al., 1976; Ogino et al., 1977; Hackstadt and Mallavia, 1978; Cheng et al., 1979). In this study, this difference was confirmed immunologically (Table 1). In previous work (Yamanishi et al., 1981), VZV was shown to induce TK on abortive infection of TK-deficient mouse cells and in this study VZV-induced TK was shown to be immunologically and electrophoretically distinct from cellular TK (Fig. 2a). Furthermore, Ltk$^-$ cells could be converted to the TK-positive phenotype by

infection with cell-associated VZV (Yamanishi et al., 1981) and the TKs expressed in these biochemically transformed cells were found to be immunologically and electrophoretically the same as those induced in cell-free VZV infected human and mouse cells (Fig. 1, 2). These results show that VZV has a coding capacity for TK in its genome.

When Ltk$^-$ cells were infected with cell-associated virus (VZV-infected HEF cells) and cultured in selective medium, TK-positive phenotype cells were obtained. In this experiment the viral gene coding for TK was suspected to be transferred into Ltk$^-$ cells, as reported for HSV (Munyon et al., 1971; 1972; Wigler et al., 1977; Maitland and McDougall, 1977; Colvere-Gorapin et al., 1979) and EHV (McGowan et al., 1979). We showed previously by chromosomal analysis that TK-positive transformed cells could be derived from mouse cells (Yamanishi et al., 1981). However, another possibility was that TK in VZV-biochemically transformed cells originated from HEF cells, because VZV-infected HEF cells were used as inoculum. This possibility was excluded by the fact that the TK activity present in biochemically transformed cells was immunologically different from that in HEF cells (Table 1), and that it was immunologically and electrophoretically the same as that induced in cell-free VZV infected human or mouse cells (Fig. 1, 2, unpublished data).

VZV-specific TK was shown to be immunologically distinct from HSV-specific TK (Table 1, 2). Herpesviruses have been shown to share common antigens. TK is an interesting functional protein to use in comparative studies on herpesviruses (Honess and Watson, 1977). In the present work, enzyme neutralization experiments with VZV- and HSV-specific antisera demonstrated that there was no cross reactivity between VZV and HSV TKs. The Rm value of HSV-induced TK was 0.7 under our conditions (data not shown). This shows that the molecular weights of the two were also different, although the molecular weight of TK was not determined in the present study. Our Rm value (0.4–0.5) for VZV-specific TK was similar to the Rm value (0.48) reported by Hackstadt and Mallavia (1978), who calculated that the molecular weight was 72,000. They mentioned in their following report that the Rm value of purified VZV-induced TK was essentially the same as that observed in the crude preparation (Cheng, Y-C. et al., 1979).

Two isozymes of TK in VZV-infected Ltk$^-$ cells were separated by poly-acrylamide gel electrophoresis, as shown in Figs. 1 and 2. However, only one peak of TK activity of Rm 0.45, corresponding to VZV TK, was detected in the transformed cells, although the peak corresponding to mitochondrial TK could be detected on prolonged incubation during the enzyme assay. The meaning of this depression of mitochondrial TK activity in VZV-transformed cells is not clear at present time. Munyon et al. (1972) reported that HSV-biochemically transformed cells had a peak at 0.70–0.80, corresponding to mitochondrial TK, but Jamieson et al. (1974) could not detect any mitochondrial TK. The lower neutralization of VZV-TK activity in Fig. 2b than that in Table 2 is considered to be due to the difference in TK assay conditions: for Fig. 2b, TK activity was assayed without removing the gel after preincubation with sera. The same extent of neutralization could be obtained when TK in gels was extracted into buffer before preincubation with sera and then the residual TK activity was assayed.

Herpesviruses, including VZV, are known to cause latent infection in vivo (Jack, 1974). Neither the biochemical nature of the latency nor the physical state of the latent virus is known at present. Our biochemically transformed cells seem to offer a good system for studies on the mechanisms involved in control of the VZV genome in mammaliam cells. TK will be useful as a marker of viral gene expression in studies on VZV infection.

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