These aspects are in accordance with a calorimetric analysis of amino acid binding to the L-isoleucine:tRNA ligase of *Escherichia coli* performed by Hinz et al. (1976). The authors found identical association enthalpies (ΔH_a) for L-Ile and a collection of its structural analogues. They demonstrated that the entropy terms (ΔS_a) are responsible for the large differences in the $\Delta G°$ of binding of these compounds.

Binding studies and kinetic experiments with substrate analogues are needed to assign the binding increments of the thermodynamic parameters to structural elements of the substrate amino acids in the biosynthesis of gramicidin S.

ACKNOWLEDGMENTS

The skillful technical assistance of R. Hacker and L. Pirling-Unger is gratefully acknowledged. We thank Professor R. Reich and Professor J. Salnikow for valuable discussions.

Registry No. GS, 37356-20-0; L-Phe, 63-91-2; L-Pro, 147-85-3; L-Val, 72-18-4; L-Orn, 70-26-8; L-Leu, 61-90-5; phenylalanine racemase, 37290-95-2.

REFERENCES

Aarstadt, K., Christiansen, C., Zimmer, T. L., & Laland, S. G. (1978) *FEBS Lett. 87*, 244–246.

Altmann, M., Koischwitz, H., Salnikow, J., & Kleinkauf, H. (1978) *FEBS Lett. 93*, 247–250.

Aust, H. J., & v. Döhren, H. (1982) in *Peptide Antibiotics—Biosynthesis and Functions* (Kleinkauf, H., & v. Döhren, H., Eds.) pp 137–148, de Gruyter, Berlin and New York.

Christiansen, C., Aarstadt, K., Zimmer, T. L., & Laland, S. G. (1977) *FEBS Lett. 81*, 121–124.

Gevers, W., Kleinkauf, H., & Lipmann, F. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 269–276.

Gevers, W., Kleinkauf, H., & Lipmann, F. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 1335–1342.

Hinz, H.-J., Weber, K., Flossdorf, J., & Kula, M.-R. (1976) *Eur. J. Biochem. 71*, 437–442.

Kanda, M., Hori, K., Kurotsu, T., Miura, S., Nozoe, A., & Saito, Y. (1978) *J. Biochem. (Tokyo) 84*, 435–441.

Katz, E., & Demain, A. L. (1977) *Bacteriol. Rev. 41*, 449–474.

Kleinkauf, H. (1979) *Planta Med. 35*, 1–18.

Kleinkauf, H., & Koischwitz, H. (1978) *Prog. Mol. Subcell. Biol. 6*, 59–112.

Kleinkauf, H., Gevers, W., & Lipmann, F. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 226–233.

Koischwitz, H., & Kleinkauf, H. (1976) *Biochim. Biophys. Acta 429*, 1041–1051.

Kurahashi, K. (1981) *Antibiotics (N.Y.) 4*, 325–352.

Laland, S. G., & Zimmer, T.-L. (1973) *Essays Biochem. 9*, 31–57.

Lipmann, F. (1973) *Acc. Chem. Res. 6*, 361–367.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Saxholm, H., Zimmer, T.-L., & Laland, S. G. (1972) *Eur. J. Biochem. 30*, 138–144.

Vater, J., & Kleinkauf, H. (1976) *Biochim. Biophys. Acta 429*, 1062–1072.

Vater, J., Mallow, N., Gerhardt, S., & Kleinkauf, H. (1982) in *Peptide Antibiotics—Biosynthesis and Functions* (Kleinkauf, H., & v. Döhren, H., Eds.) pp 219–231, de Gruyter, Berlin and New York.

Warburg, O., & Christian, W. (1941) *Biochem. Z. 310*, 384–421.

Yamada, M., & Kurahashi, K. (1969) *J. Biochem. (Tokyo) 66*, 529–540.

Epstein–Barr Virus Induces a Unique Pyrimidine Deoxynucleoside Kinase Activity in Superinfected and Virus-Producer B Cell Lines†

Timothy Stinchcombe and Wendy Clough*

Molecular Biology Section, Department of Biological Sciences, University of Southern California, Los Angeles, California 90089

Received August 8, 1984

ABSTRACT: Epstein–Barr (EB) virus induces a new pyrimidine deoxynucleoside kinase [thymidine kinase (dTk)] activity in Raji B lymphocyte cells after superinfection. This dTk activity is also present in small amounts in the HR-1 virus-producer cell line and in larger amounts in the B95-8 virus-producer line. The dTk activity induced by EB virus coelutes from DEAE-cellulose columns with deoxycytidine kinase (dCk) activity and elutes as a broad peak well separated from the large peaks of cellular dTk and dCk activities. This EB virus-induced pyrimidine deoxynucleoside kinase activity from HR-1 cells differs from cellular kinases in most basic biochemical properties but shares certain properties with the herpes simplex virus dTk.

Herpes viruses induce most of the enzymes required for replication of viral DNA in productively infected cells. These viral enzymes include DNA polymerase (Weissbach et al., 1973; Boezi et al., 1974; Huang, 1975; Allen et al., 1977; Goodman et al., 1978), DNase (Hoffman & Cheng, 1978; Clough, 1979, 1980; Hoffman, 1981), and a recently reported topoisomerase (Biswal et al., 1983). Virus-induced pyrimidine deoxynucleoside kinase [thymidine kinase (dTk)][1] activity has been detected in cells productively infected with a variety of herpes viruses, including herpes simplex virus (Ogino et al., 1973; Jamieson & Subak-Sharpe, 1974), varicella-zoster virus (Ogino et al., 1977), and herpes virus saimiri (Honess et al.,

†This work was supported by National Institutes of Health Grant CA23070 and Research Career Development Award CA00707.

[1] Abbreviations: EB virus, Epstein–Barr virus; dTk, thymidine kinase; dCk, deoxycytidine kinase; dT, thymidine; dC, deoxycytidine; TPA, 12-*O*-tetradecanoylphorbal 13-acetate; dTk$^-$, dTk negative; DEAE, diethylaminoethyl; PMSF, phenylmethanesulfonyl fluoride; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; K_2PO_4, mixture of mono- and dipotassium phosphate salts adjusted to pH 8.0.

0006-2960/85/0424-2027$01.50/0

1982). Unlike DNA polymerases and nucleases, thymidine kinases both cellular and viral have been highly resistant to purification using conventional column chromatography techniques.

The role of thymidine kinase in herpes virus DNA replication is of interest because nucleoside analogues such as acyclovir [9-[(2-hydroxyethoxy)methyl]guanine] are effective inhibitors of viral replication only after phosphorylation of the compound by the viral-specific dTk. In contrast to herpes simplex virus, Epstein–Barr virus (EB virus) has not yet been associated with a viral dTk activity. However, much circumstantial evidence supports the existence of such a viral activity in EB virus-producing cells. Glaser et al. (1973) reported alteration of dTk activity in Burkitt lymphoma crude cell extracts after EB virus induction. Recently, it has been reported that 1-β-D-arabinofuranosylthymine is a selective inhibitor of EB virus replication (Ooka et al., 1983). Crude extracts obtained from Raji dTk-negative (dTk^-) cells superinfected with EB virus from P3HR-1 cells demonstrate a dTk activity not found in normal Raji dTk^- cells (Roubal & Klein, 1981). Furthermore, it has been reported that [^{3}H]-thymidine is incorporated into bromodeoxyuridine-resistant, dTk^- P3HR-1, and Raji cells after the onset of the viral replication cycle (Hampar et al., 1971, 1972). Treatment of P3HR-1 dTk^- cells with the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and sodium butyrate also induces high [^{3}H]thymidine incorporation into EB virus DNA (Ooka & Calender, 1980). Sera from patients with nasopharyngeal carcinoma containing a high antibody titer against EB virus early antigen have the ability to inactivate a dTk activity found in crude extracts of Raji and NC37 dTk^- cells treated with TPA or *n*-butyrate or superinfected with the P3HR-1 strain of EB virus (MacGabhann et al., 1984). However, crude cell extracts contain large amounts of cellular thymidine kinase and deoxycytidine kinase activities, and an EB virus-specific dTk activity has not been separated from cellular enzymes and characterized.

We report here the isolation and characterization of a unique EB virus-induced dTk activity. As is the case of EB virus-induced nuclease and DNA polymerase, this EB virus-specific dTk activity is found in B cell lines that productively replicate viral DNA and in superinfected non-virus-producer Raji cells, but not in untreated nonproducer cells. This virus-induced dTk possesses biochemical properties distinctly different from host cell cytosol or mitochondrial dTk activities and has some properties similar to those of the herpes simplex virus dTk.

Experimental Procedures

Materials. [*methyl*-^{3}H]Thymidine (72 Ci/mmol) and deoxy[5-^{3}H]cytidine (20 Ci/mmol) were from ICN. Nonradioactive deoxynucleotides, thymidine (dT), deoxycytidine (dC), Nonidet P-40 (NP-40), phenylmethanesulfonyl fluoride (PMSF), and TPA were from Sigma. DEAE-52 cellulose and DEAE filter paper were from Whatman. RPMI 1640 medium and fetal calf serum were from Gibco.

Cells. Lymphocyte cell lines used included B95-8 and Raji which have been described elsewhere (Clough, 1979). HR-1 and Raji dTk^- cell lines were obtained from Dr. Nonoyama. The HR-1 cell line which is a derivative of P3HR-1 has previously been described (Thorley-Lawson, 1979). Raji dTk^- cells are similar to normal Raji cells except that thymidine kinase activity is not expressed. Cell culture was performed as described elsewhere (Clough, 1979).

DEAE-cellulose Column Chromatography. Procedures for harvesting cells, making crude extracts, and performing DEAE-cellulose column chromatography have been previously described by Goodman et al. (1978) and Clough (1979). Briefly, modifications of the published procedures included harvesting of 1.0 L of cells 96 h after feeding to 5.0×10^5 cells/mL for routine dTk and dCk isolation. With this number of cells, passage through only one DEAE-cellulose column (17 mm × 110 mm) was necessary to obtain separation of pyrimidine deoxynucleoside kinase activities. The column buffer was 20 mM K_2PO_4, pH 8.0, that included 20% glycerol, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.2% NP-40, and 10 μg of PMSF/mL. A 100-mL gradient of 20–400 mM K_2PO_4 buffer [see Clough (1979)] was sufficient to allow elution of bound proteins; 2-mL fractions were collected.

Pyrimidine Deoxynucleoside Kinase Assay. Thymidine kinase and deoxycytidine kinase activity was measured by using a modification of the DE81 disk method described by Jamieson & Subak-Sharpe (1974). The modification included an assay buffer consisting of 50 mM Tris·HCl (pH 7.0 at 37 °C), 5 mM $MgCl_2$, 10 mM NaF, 1 mM ATP, and 30 μL of [^{3}H]dT/mL of assay mix. For deoxycytidine kinase activity, 30 μL of [^{3}H]dC/mL of assay buffer was substituted for [^{3}H]dT. The reaction mix consisted of 25 μL of sample and 75 μL of assay buffer. After 60 min at 37 °C, 50 μL of reaction mixture was spotted onto 24-mm-diameter DE81 disks. The disks were washed twice with 5 mM ammonium formate, once with distilled water, and finally with absolute ethanol. All washes were for 15 min. After being dried, the filters were counted in a Beckman LS-230 scintillation counter.

One unit of activity was defined as 1 pmol of dT or dC phosphorylated in 1 h at 37 °C under the above assay conditions. One unit typically represented 50 000 cpm with [^{3}H]dT and 25 000 cpm with [^{3}H]dC.

Raji Superinfection. Superinfection of Raji or Raji dTk^- cells with virus obtained from HR-1 cell culture has been described previously (Grossberger & Clough, 1981). When radioactive labeling of DNA was necessary, 1 μCi of [^{3}H]-dT/mL of cell culture was added at the time of superinfection. Isolation of [^{3}H]dT-labeled DNA by CsCl equilibrium centrifugation was described previously (Grossberger & Clough, 1981). For DEAE-cellulose chromatography (Figure 1), 2.5×10^8 cells were superinfected and used to make crude extracts whereas 4.0×10^7 superinfected cells were used for isolation of [^{3}H]dT-labeled DNA (Figure 2).

Induction with TPA. Induction of viral replication with the tumor promoter TPA has been described elsewhere (Hudewentz et al., 1980). The final concentration of TPA was 20 ng/mL. Cells were fed to 5.0×10^5/mL and TPA treated 1 day later. Cells were harvested at 4 days posttreatment.

Results

In the case of EB virus-induced DNA polymerase and nuclease, it has been shown that nonproducer Raji cells contain only host enzymatic activities and that virus-induced enzymes appear in Raji cells only after EB virus superinfection or drug treatment (Goodman et al., 1978; Clough, 1979; Feighny et al., 1980; Ooka & Calender, 1980; Clough & McMahon, 1981). Also, EB virus-induced enzymes are found in viral capsid antigen-positive producer cell populations, namely, HR-1 and B95-8 cell lines (Goodman et al., 1978; Clough, 1979). Therefore, we postulated that any EB virus-induced pyrimidine deoxynucleoside kinase activity would be induced in Raji cells by superinfection and would be present in EB virus-producer cell lines. In order to separate and identify pyrimidine deoxynucleoside kinase activities present in EB virus-positive B cell lines, cell extracts were chromatographed in DEAE-cellulose columns as previously described (see Experimental Procedures), using superinfected Raji cells and

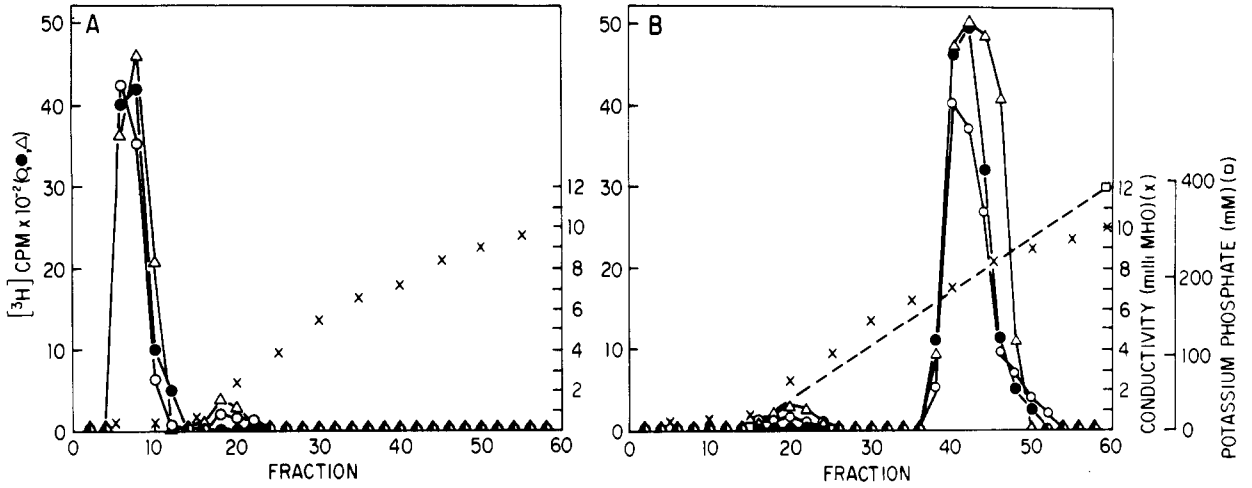

FIGURE 1: Pyrimidine deoxynucleoside kinase activities in Raji cells at various times after superinfection with HR-1 virus. Preparations of Raji cells containing of total of 2.5×10^8 cells each were superinfected [see Experimental Procedures and Grossberger & Clough (1981)], and cell extracts were passed through DEAE-cellulose columns [see Experimental Procedures and Clough (1979)]. Alternate column fractions were tested for (A) thymidine kinase or (B) deoxycytidine kinase. Harvesting of cells post-superinfection occurred at (●) 3.0, (○) 7.0, and (△) 20.0 h. The summed counts from assays of column fractions for each activity peak are shown below. (For both dTk and dCk, alternate fractions were assayed and only these numbers added. The actual cpm would therefore be approximately double those shown above had every fraction been assayed for each activity.)

	activity (cpm) for			
superinfected Raji cells (h)	dTk at 20 mM K_2PO_4	dTk at 40–60 mM K_2PO_4	dCk at 40–60 mM K_2PO_4	dCk at 250–350 mM K_2PO_4
3.0	9700	0	0	15 625
7.0	8255	905	940	14 775
20.0	8140	1235	1310	20 050

virus-producer cells with and without drug stimulation.

Pyrimidine Deoxynucleoside Kinase Activities Present in Superinfected Raji Cells. At various times after superinfection (3, 7, and 20 h), Raji cells were harvested; cell extracts were subjected to DEAE-cellulose chromatography, and the fractions obtained were tested for pyrimidine deoxynucleoside kinase activity. As shown in Figure 1A, a single dTk activity eluting at 40–60 mM K_2PO_4, pH 8.0, buffer appears between 3- and 7-h post-superinfection and is present in greater amounts at 20-h post-superinfection. A second dTk activity that elutes in the void volume (20 mM K_2PO_4) is present throughout the course of superinfection.

Dobersen & Greer (1978) have shown that the herpes simplex virus dTk has the ability to phosphorylate not only thymidine but also deoxycytidine, unlike host cytosol thymidine kinase which phosphorylates only thymidine. When fractions from DEAE-cellulose columns of Raji superinfected cells were tested for deoxycytidine kinase (dCk), two activities were present as depicted in Figure 1B. One activity, which eluted at 40–60 mM K_2PO_4, appeared after 3-h post-superinfection. This dCk activity, which coelutes with the induced dTk activity, was also present in greater amounts at 20-h than at 7-h post-superinfection. A second dCk activity eluted at 250–350 mM K_2PO_4 and was present throughout the course of Raji superinfection.

[^{3}H]Thymidine Incorporation into Epstein–Barr Viral DNA during Superinfection of Raji dTk$^-$ Cells. To confirm and expand upon the results presented in Figure 1, superinfected cells can be examined in other ways. For example, Roubal & Klein (1981) reported that crude extracts prepared from superinfected Raji dTk$^-$ cells showed dTk activity that was maximal by 24-h postinfection. The Raji dTk$^-$ cell line differs from its parent Raji cell line only in that it lacks dTk activity and exogenous thymidine is not utilized in significant amounts in DNA replication. We have shown that the Raji dTk$^-$ cell line has no detectable dTk activity (unpublished results).

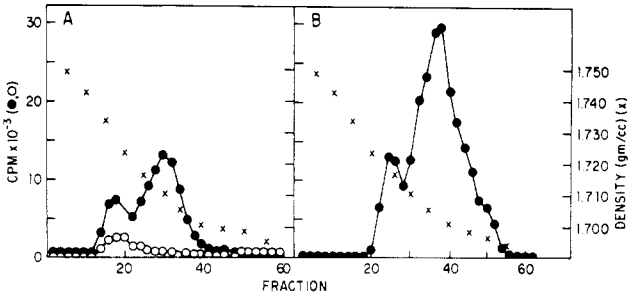

FIGURE 2: Isolation of [^{3}H]dT-labeled DNA from Raji and Raji dTk$^-$ superinfected cells by cesium chloride equilibrium ultracentrifugation. Superinfection, ultracentrifugation, and [^{3}H]dT labeling were as described under Experimental Procedures or by Grossberger & Clough (1981). DNA was isolated from Raji dTk$^-$ superinfected (●) or mock-superinfected (○) cells (A) and from normal Raji superinfected cells (●) (B).

However, the data in Figure 2 show that large amounts of [^{3}H]dT are incoporated into viral as well as cellular DNA after superinfection of Raji dTk$^-$ cells with EB virus.

[^{3}H]dT was added during superinfection of Raji dTk$^-$ cells with HR-1 virions as described under Experimental Procedures. At 20-h post-superinfection, DNA from the Raji dTk$^-$ cells was isolated and sedimented in CsCl density gradients. Incorporation of [^{3}H]dT into viral and host DNA is shown in Figure 2A. Two peaks of labeled DNA are present in the case of superinfected Raji dTk$^-$ cells. One peak of density 1.718 g/cm^3 represents viral DNA while a second peak of density 1.710 g/cm^3 is characteristic of host cell DNA (Schulte-Holthausen & zur Hausen, 1970). In contrast, mock superinfection of Raji dTk$^-$ cells is followed by only trace amounts of [^{3}H]dT incorporation into DNA of either density. Raji cell superinfection with HR-1 virions serves as a control to demonstrate [^{3}H]dT incorporation into viral DNA as described by Roubal & Klein (1981). [^{3}H]dT incorporation in ordinary Raji cells is shown in Figure 2B and is similar to

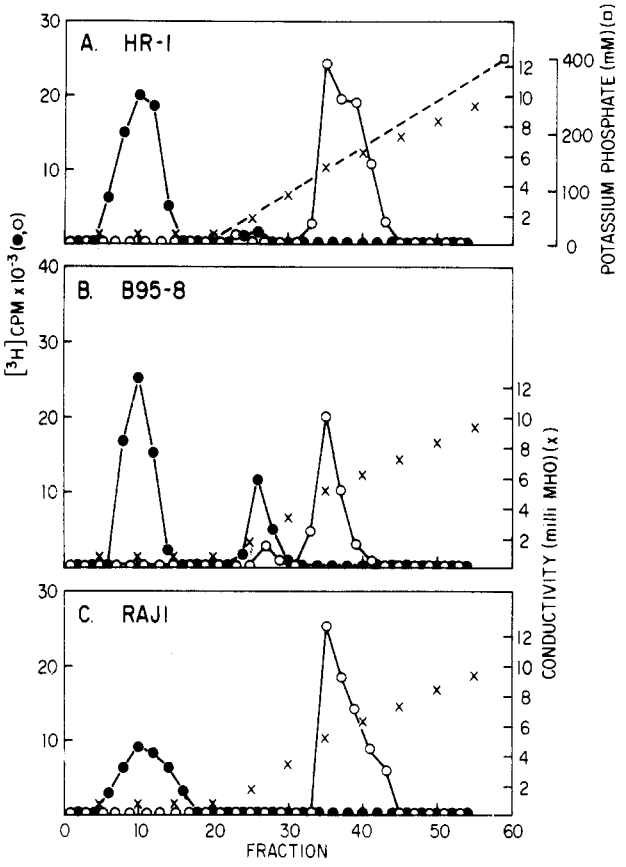

FIGURE 3: DEAE-cellulose chromatography of cell extracts from various cell lines. Extracts were prepared as described under Experimental Procedures and by Goodman et al. (1978) and Clough (1979) and passed through DEAE-cellulose columns, and alternate fractions were tested for thymidine kinase (●) or deoxycytidine kinase (○) activity. (A) Cell extract from 1.0 L of HR-1 cells; (B) cell extract from 1.0 L of B95-8 cells; (C) cell extract from 1.0 L of Raji cells.

	total protein loaded (μg)	total dTk + dCk units loaded	total dTk + dCk units recovered
HR-1	271	1925	1505
B95-8	278	2015	1095
Raji	315	1630	1240

	activity (cpm) for dTk at 20 mM K_2PO_4	dTk at 40–60 mM K_2PO_4	dCk at 40–60 mM K_2PO_4	dCk at 250–350 mM K_2PO_4
HR-1	64 600	3130	3735	79 695
B95-8	59 680	22090	3760	40 830
Raji	24 835	0	0	84 345

(For both dTk and dCk, alternate fractions were assayed and only these numbers added. The actual total counts would therefore be approximately double those shown above had every fraction been assayed for each activity.)

[^{3}H]dT incorporation in Raji dTk$^-$ superinfected cells.

These results are in agreement with the data shown in Figure 1 and with the finding of Roubal & Klein (1981) that new dTk activity appears in dTk$^-$ cells after viral superinfection.

Pyrimidine Deoxynucleoside Kinase Activities Associated with B95-8, HR-1, and Raji B Cell Lines. The fractions from DEAE-cellulose chromatograms of HR-1 and B95-8 cells (both virus-producer cell lines) contain two dTk activities as shown in Figure 3A,B. One dTk activity elutes in the column void volume (20 mM K_2PO_4), and the second elutes at 40–60 mM K_2PO_4. The Raji (nonproducer) cell line demonstrates only one dTk activity which elutes in the column void volume

Table I: Pyrimidine Deoxynucleoside Kinase Activities in TPA-Treated As Compared to Untreated HR-1 Cells[a]

elution from DEAE-cellulose	type of activity	units of activity[b] +TPA	units of activity[b] −TPA	+TPA/−TPA ratio
40–60 mM K_2PO_4 buffer	dTk	39.8	16.9	2.4
	dCk	3.9	1.0	3.9
20 mM K_2PO_4 (void volume)	dTk	59.3	55.7	1.1
250–350 mM K_2PO_4	dCk	123.2	281.4	0.4

[a] One liter of HR-1 cells was treated with 20 ng of TPA/mL 1 day after feeding to 5×10^5 cells/mL and harvested 4 days later. Control cultures were treated identically but not exposed to TPA. Cell extracts were chromatographed through DEAE-cellulose columns and assayed for dTk and dCk activities (see Experimental Procedures and legends to Figures 1 and 3). [b] One unit of pyrimidine deoxynucleoside kinase activity is defind as 1 pmol of [^{3}H]dT or [^{3}H]dC phosphorylated in 1 h at 37 °C (50 760 cpm for dT; 25 040 cpm for dCd).

(20 mM K_2PO_4) (Figure 3C). Column fractions were also assayed for dCk activity as depicted in Figure 3. All cell lines tested demonstrated a dCk activity which elutes at 250–350 mM K_2PO_4. A second small peak of dCk activity is present in the HR-1 and B95-8 producer cell lines and coelutes with the thymidine kinase activity found in fractions representing 40–60 mM K_2PO_4.

In the case of HR-1 cells, the dTk activity eluting at 40–60 mM K_2PO_4 frequently comprised 5% or less of total cellular dTk. In marked contrast, in B95-8 cells, usually greater than 25% of total cellular dTk was the material eluting at 40–60 mM K_2PO_4. It is also important to note that all peaks of dTk and dCk activities as shown in Figure 3 were subjected to rechromatography through DEAE-cellulose columns. In all cases, all material eluted exactly as it had on the initial chromatography (data not shown).

To determine if any of the dTk activities isolated were of mitochondrial origin, HR-1 mitochondria were prepared as described by Kit et al. (1973), and the mitochondrial extract was subjected to DEAE-cellulose chromatography. The fractions collected were then assayed for dTk activities. A single mitochondrial dTk activity elutes in the void volume at 20 mM K_2PO_4 (data not shown).

Pyrimidine Deoxynucleoside Kinase Activities Associated with EB Virus Replication Induced by Treatment with TPA. EB viral DNA replication is stimulated by treatment of producer cell lines with TPA (Hudewentz et al., 1980; Ooka & Calender, 1980). TPA treatment also increases the amount of viral DNA polymerase in producer cells (Datta et al., 1980a,b) while decreasing or leaving unchanged levels of host cell enzymes. We wished to determine if levels of any of the pyrimidine deoxynucleoside kinase (dTk or dCk) activities found in fractions from DEAE-cellulose chromatograms of producer HR-1 cells changed with TPA stimulation.

HR-1 cells were treated with TPA (or untreated for controls) 1 day after feeding and harvested 4 days later, and cell extracts were chromatographed through DEAE-cellulose columns. As shown in Table I, the amount of dTk activity found in the void volume (20 mM K_2PO_4) from HR-1 cell extracts does not change significantly during 4 days of TPA stimulation, but the dTk activity eluting at 40–60 mM K_2PO_4 increases 2.4-fold over the same activity in unstimulated cells. The dCk activity coeluting with dTk at 40–60 mM K_2PO_4 increased 4-fold with TPA stimulation while the dCk activity eluting at 250–350 mM K_2PO_4 has decreased in amount in TPA-stimulated as compared to unstimulated cells. The dTk/dCk ratio is different in the peak of virus-induced activity

Table II: Summary of Properties of HR-1 Pyrimidine Deoxynucleoside Kinases

	EB virus-induced activity at 40–60 mM K_2PO_4	cytosol dTk activity at 20 mM K_2PO_4	cytosol dCk activity at 250–350 mM K_2PO_4
pH optima[a]			
dT substrate	6.5–7.0	7.5	
dC substrate	7.5		8.0
K_m[b] (μM)			
dT substrate	0.8	2.3	
dC substrate	0.2		0.5
Mg^{2+} requirement[a] of dT substrate (mM)	2–8	8–10	
TTP inhibn (200 μM)[c] (%)			
dT substrate	18 remaining act.	14	
dC substrate	100		135
dCTP inhibn (200 μM) (%)			
dT substrate	81	80	
dC substrate	50		2

[a] Assays to determine pH optima used the method described under Experimental Procedures except that either the pH of the Tris buffer or the concentration of $MgCl_2$ was varied. [b] Assays for the K_m determination were performed by using the procedure described under Experimental Procedures except that they were terminated after 15-min incubation at 37 °C. K_m values were then obtained from double-reciprocal plots of velocity vs. substrate concentration. [c] Inhibition assays were performed as described under Experimental Procedures except that assays were terminated after 15 min. TTP or dCTP was added at concentrations ranging from 0 to 500 μM. Shown are values for the percent activity remaining in the presence of 200 μM TTP or dCTP. 100% activity values were between 2400 and 4400 cpm per assay.

in Figure 3 and in Table I. In fact, the dTk/dCk ratio was variable in all of our chromatographic data; while we cannot explain the variability of the ratio at this time, it suggests that the two activities may be separate rather than a single active site on a single molecule (also see TTP inhibition data in Table II).

Biochemical Differences among Pyrimidine Deoxynucleoside Kinases Isolated by DEAE-cellulose Chromatography of HR-1 Cell Extracts. Biochemical studies were performed on those fractions exhibiting dTk or dCk activities obtained from DEAE-cellulose chromatograms of HR-1 cell extracts. In spite of the much smaller amount of EB virus-induced dTk activity present in HR-1 as compared to B95-8 producer cells, pyrimidine deoxynucleoside kinase activities from HR-1 cells were used for these characterization experiments because (1) HR-1 virus was used in the Raji superinfection experiments shown in Figures 1 and 2 and (2) characterization of other EB virus-induced enzymes was performed with material isolated from HR-1 cells (Goodman et al., 1978; Clough, 1979, 1980). Fractions containing the dTk activity eluting with 20 mM K_2PO_4 in the void volume were pooled and considered to represent cellular or cytosol dTk. Column fractions containing dTk and dCk activity eluting with 40–60 mM K_2PO_4 were pooled and designated as EB virus specific since this activity increased with TPA stimulation of HR-1 cells or appeared during Raji superinfection. Fractions which contained a single dCk activity eluting at 250–350 mM K_2PO_4 were pooled and considered host cell or cytosol dCk since this activity was not correlated with active viral production (Figures 1 and 3). The following data are presented in Table II.

When the effect of pH on enzyme activity was measured, it was found that the optimum pH for the HR-1 EB virus-specific dTk was 6.5–7.0 whereas the host cytosol dTk exhibited an optimum pH of 7.5. The EB virus-specific dTk-containing fractions also contained a dCk activity with a pH optimum of 7.5, whereas the host cytosol dCk has a pH optimum of 8.0.

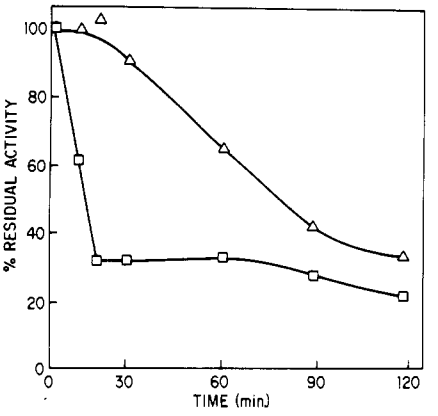

FIGURE 4: Inactivation of host cytosol dTk and EB virus-induced dTk by incubation at 45 °C for various times. Kinase activities were obtained by pooling fractions from HR-1 DEAE-cellulose columns such as that depicted in Figure 3A. Host cytosol dTk activity (at 20 mM K_2PO_4) (△); EB virus-induced dTk activity (at 40–60 mM K_2PO_4) (□).

The K_m values for the host cytosol and EB virus-specific dTk and dCk activities were determined by using double-reciprocal plots of velocity against substrate concentration, and the resultant values are shown in Table II. The K_m for the HR-1 EB virus-specific dTk with thymidine substrate was 0.8 μM and with deoxycytidine was 0.2 μM. The K_m for host cytosol dTk was 2.3 μM and for the host dCk was 0.5 μM. Table II also shows that optimal Mg^{2+} concentration for the EB virus-specific dTk was in the range of 2–8 μM but was higher (8–10 μM) for the cellular dTk.

The EB virus-induced dTk was strongly inhibited by 200 μM TTP (18% activity remaining) but was only slightly inhibited by 200 μM dCTP (81% activity remaining). The cytosol dTk behaved in a similar manner in the presence of these inhibitors. Interestingly, the EB virus-specific kinase assayed in the presence of deoxycytidine as substrate was completely resistant to 200 μM dTTP added as inhibitor but was inhibited by 50% by 200 μM dCTP, in contrast to the herpes simplex virus dTk (Dobersen & Greer, 1978) and suggesting that in the case of the EB virus-induced kinase the two activities may occupy separate sites or even separate molecules.

The herpes simplex virus dTk has been shown to be more sensitive to heat inactivation than its corresponding host cell activity. To test the stability of the kinase activities, a variety of conditions were imposed. Samples were heated to 45 °C and were tested at various times to determine the amount of residual activity present. The data in Figure 4 show that the EB virus-specific dTk is markedly more susceptible to heat inactivation than the cellular dTk. Only 30% of the original EB virus-specific dTk is present after treatment at 45 °C for 15 min, whereas 98% of the cellular dTk activity remains.

The EB virus-specific dTk was stable at 5 °C only under certain conditions. If dialyzed against dTk assay buffer in the absence of phenylmethanesulfonyl fluoride (10 μg/mL) or 2-mercaptoethanol (5 mM), only 10% of the original activity remained after 4 h; this was found to be a higher level of dependence on mercaptoethanol than that exhibited by cellular dTk or dCk activities. Inclusion of these two enzyme stabilizers resulted in retention of 90% of the original viral dTk activity after 24 h. (PMSF and 2-mercaptoethanol were routinely included in all buffers and assays.) Under these optimal conditions for stability, the EB virus-induced dTk was able to continue to phosphorylate thymidine or deoxycytidine

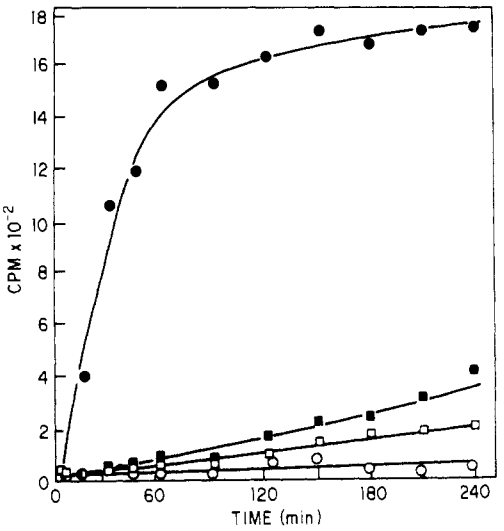

FIGURE 5: Phosphorylation of thymidine with time. Host cytosol dTk and EB virus-induced dTk were obtained by pooling fractions from HR-1 DEAE-cellulose columns as previously described. At the times indicated, 50-μL samples of reaction mix were spotted onto DEAE paper disks, and the disks were washed and counted as described under Experimental Procedures. Host cytosol dTk (●); EB virus-induced dTk (■); host cytosol dCk (□); buffer control (○).

Table III: Relative Nucleoside Triphosphate Usage by dTk Activities from HR-1 Cells[a]

phosphate donor (10 mM)	EB virus-induced activity at 40–60 mM K_2PO_4 (%)	cytosol dTk activity at 20 mM K_2PO_4 (%)
ATP	100	100
dATP	61	99
UTP	61	4
GTP	59	3
dGTP	46	13
CTP	30	14
dCTP	20	14
TTP	0	0

[a] The final concentration (10 mM) of the nucleotide phosphate donors indicated in column 1 was substituted for ATP in the assay mix described under Experimental Procedures. The activity with ATP as phosphate donor was taken as 100%.

for at least 4 h at 37 °C (the standard assay temperature). In contrast, the cellular dTk ceased phosphorylating thymidine by 120 min of incubation under the same conditions, although greater than 90% of substrate and phosphate donor were still available (Figure 5).

The nucleoside triphosphate usage as phosphate donor by the EB virus-induced dTk is compared to that of the host cytosol dTk in Table III. In both cases, ATP was the preferred triphosphate, and TTP at 10 mM was inhibitory and unable to act as phosphate donor; otherwise, as in the other biochemical analyses, the virus-induced and cytosol dTk's show marked differences in phosphate donor preferences.

Discussion

The results presented show that two dTk activities exist in EB virus-producing cell lines, eluting as separate activities from DEAE-cellulose columns and showing marked differences upon biochemical characterization. One of these dTk activities is correlated with active viral DNA replication. The same is true for two distinct, separable dCk activities, one of which coelutes from DEAE-cellulose columns with a dTk activity. The coeluting dCk and dTk activities are considered virus specific or induced because, as in the case of viral polymerase and nuclease, they appear in increasing amounts during superinfection of Raji cells and TPA stimulation of HR-1 cells, are present in virus-producer cells, and are not present in virus-nonproducer cells.

The EB virus-specific dTk activity described here resembles the herpes simplex virus dTk activity in certain biochemical characteristics. These include a marked temperature sensitivity at 45 °C, a K_m with thymidine as substrate of 0.8 μM, the ability to phosphorylate both thymidine and deoxycytidine, and the ability to utilize a variety of nucleoside triphosphates as phosphate donor.

To date, the evidence for an EB virus-specific dTk has been derived from studies with crude cell extracts showing changing dTk activities associated with EB viral DNA replication (Ooka et al., 1983; Roubal & Klein, 1981; Hampar et al., 1971, 1972) or inhibition of EB virus replication by nucleoside analogues such as acyclovir (Datta et al., 1980a,b). However, until now, no group has yet reported separation or purification of host cytosol dTk from an EB virus dTk. Our data demonstrate two difficulties that could have prevented separation of virus-induced and cellular kinases: (1) the virus-specific activity is, in the case of HR-1 or superinfected Raji, a very small percentage of the total pyrimidine deoxynucleoside kinase activity within producer cells; and (2) the virus-induced kinase is inactivated rapidly at 5 °C in the absence of 2-mercaptoethanol and PMSF.

Our data show that in cells containing the EB virus-induced dTk activity, significant amounts of cellular dTk and dCk activities remain, even after superinfection of Raji cells or stimulation of HR-1 cells with TPA; in fact, cellular kinase activities are the predominant species. This indicates that crude cell extracts are unsuitable for EB virus, dTk or dCk studies beyond preliminary observations.

Pyrimidine deoxynucleoside kinases from mammalian cells have not been amenable to purification in significant amounts using conventional column chromatography techniques. One notable exception is the purification of human liver dTk using thymidine affinity chromatography (Ellims & van der Weyden, 1980). However, 100 g of liver was used as starting material. Since the EB virus-specific dTk in B lymphocyte cell lines is present in small amounts, attempts at further purification are not feasible unless large amounts of a cloned gene product should become available.

Studies by Jamieson & Subak-Sharpe (1974) of herpes simplex type I virus dTk led to the conclusion that the active site on this enzyme allowed phosphorylation of both thymidine and deoxycytidine although kinetic studies indicated that there is probably more than one allosteric site. In the case of the EB virus-induced dTk, the variability of the dTk/dCk activity ratios and the inability of TTP to inhibit the dCk portion of the activity or of dCTP to inhibit the dTk activity suggest that more than one active site is involved. Further purification of the viral enzyme described here is necessary to determine whether the dTk and dCk activities are one or two coeluting protein species and what relationship dTk and dCk activities bear to phosphorylation of other nucleosides. Two approaches are in progress to solve the purification problem: (1) we are attempting to identify a specific cloned EB virus DNA restriction fragment that codes for the enzymatic activity; and (2) we are undertaking monoclonal antibody development in an effort to design better affinity column chromatography procedures.

Dobersen & Greer (1978) showed that the herpes simplex virus dTk has the ability to phosphorylate 5-bromodeoxycytidine, and Shaeffer et al. (1978) demonstrated acyclovir phosphorylation by a herpes-specific dTk. For future studies,

it is important to determine which nucleoside analogues can be phosphorylated by the EB virus-specific dTk, since such analogues may be suitable for clinical treatment of EB virus infections as well as herpes simplex infections.

Registry No. dTk, 9002-06-6; dCk, 9039-45-6; pyrimidine deoxyribonucleoside kinase, 55354-48-8.

REFERENCES

Allen, G. P., O'Callaghan, D. J., & Randall, C. C. (1977) *Virology 76*, 395–408.

Biswal, N., Feldan, P., & Levy, C. C. (1983) *Biochim. Biophys. Acta 740*, 379–389.

Boezi, J. A., Lee, L. F., Blakesley, R. W., Koenig, M., & Towle, H. C. (1974) *J. Virol. 14*, 1209–1219.

Clough, W. (1979) *Biochemistry 18*, 4517–4521.

Clough, W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6194–6198.

Clough, W., & McMahon, J. (1981) *Biochim. Biophys. Acta 656*, 76–85.

Datta, A. K., Colby, B. U., Shaw, J. E., & Pagano, J. S. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5163–5166.

Datta, A. K., Feighny, R. J., & Pagano, J. S. (1980b) *J. Biol. Chem. 255*, 5120–5125.

Dobersen, J. M., & Greer, S. (1978) *Biochemistry 17*, 920–928.

Ellims, P. H., & Van Der Weyden, M. B. (1980) *J. Biol. Chem. 255*, 11290–11295.

Feighny, R. J., Henry, B. E., Datta, A. K., & Pagano, J. S. (1980) *Virology 107*, 415–423.

Glaser, R., Ogino, T., Zimmerman, J., & Rapp, F. (1973) *Proc. Soc. Exp. Biol. Med. 142*, 1059–1062.

Goodman, S. R., Prezyna, C., & Benz, W. C. (1978) *J. Biol. Chem. 253*, 8617–8628.

Grossberger, D., & Clough, W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7271–7275.

Hampar, B., Derge, J. G., Martos, L. M., & Walker, J. L. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 3185–3189.

Hampar, B., Derge, J. G., Martos, L. M., & Walker, J. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 78–82.

Hoffman, P. J. (1981) *J. Virol. 38*, 1005–1014.

Hoffman, P. J., & Chen, Y.-C. (1978) *J. Biol. Chem. 253*, 3557–3562.

Honess, R. W., O'Hare, P., & Yound, D. (1982) *J. Gen. Virol. 58*, 237–249.

Huang, E. S. (1975) *J. Virol. 16*, 298–310.

Hudewentz, J., Bornkamm, G. W., & zur Hausen, H. H. (1980) *Virology 100*, 175–178.

Jamieson, A. T., & Subak-Sharpe, J. H. (1974) *J. Gen. Virol. 24*, 481–492.

Kit, S., Leung, W. C., & Trkula, D. (1973) *Arch. Biochem. Biophys. 158*, 503–513.

MacGabhann, P., Sugawara, K., & Ito, Y. (1984) *Intervirology 21*, 104–109.

Ogino, T., Shiman, R., & Rapp, F. (1973) *Intervirology 1*, 80–95.

Ogino, T., Otsuka, T., & Takahashi, M. (1977) *J. Virol. 21*, 1232–1235.

Ooka, T., & Calender, A. (1980) *Virology 104*, 219–233.

Ooka, T., Calender, A., DeTurenna, M., & Daillie, J. (1983) *J. Virol. 46*, 187–195.

Roubal, J., & Klein, G. (1981) *Intervirology 15*, 43–48.

Schaeffer, H. J., Beauchamp, L., de Miranda, P., Elion, G. B., Bauer, D. J., & Collins, P. (1978) *Nature (London) 272*, 583–585.

Schulte-Holthausen, H., & zur Hausen, H. (1970) *Virology 40*, 776–779.

Thorley-Lawson, D. (1979) *Cell (Cambridge, Mass.) 16*, 33–42.

Weissbach, A., Hong, S.-C. L., Aucker, I., & Muller, R. (1973) *J. Biol. Chem. 248*, 6270–6277.