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# Characterization of the Epstein-Barr Virus-Encoded Thymidine Kinase Expressed in Heterologous Eucaryotic and Procaryotic Systems

EDWARD LITTLER* AND JOHN R. ARRAND

*Department of Molecular Biology, Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Withington, Manchester, M20 9BX, United Kingdom*

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**The establishment of mammalian and procaryotic systems which express the Epstein-Barr virus (EBV) thymidine kinase (TK) has been reported previously (E. Littler, J. Zeuthen, A. A. McBride, E. Trøst-Sørensen, K. L. Powell, J. E. Walsh-Arrand, and J. R. Arrand, EMBO J. 5:1959–1966, 1986). The EBV TK activity expressed in both of these systems was characterized by in vitro assays and found to resemble that of the herpes simplex virus TK both in its broad range of nucleoside and nucleotide utilization and also in its ability to accept antiviral nucleoside analogs as substrates. Further results are presented which suggest that these in vitro systems may prove suitable for studying the potential anti-EBV activity of other candidate antiviral compounds.**

All the human herpesviruses, except human cytomegalovirus, have been shown to induce a novel thymidine kinase (TK) activity in virus-infected cells (3, 8).

Although the biochemical and genetic characteristics of the herpes simplex virus (HSV) TK are well established, the properties of the Epstein-Barr virus (EBV)-encoded TK are relatively poorly understood. In particular, although examination of EBV-infected lymphoblastoid cells has shown the presence of a novel TK (2, 5, 17, 19), the relatively low level of EBV-encoded proteins in such systems has hindered detailed examination of the nature of the enzyme, and definitive evidence for the viral origin of the new TK activity has remained elusive.

Recently, more compelling evidence that EBV codes for a TK has been obtained by (i) production of mammalian cell lines transformed to a $TK^+$ phenotype by EBV genomic and subgenomic DNA, (ii) detection in the transformants of a new protein related immunologically to HSV TK, (iii) construction of recombinant plasmids which express the BXLFI open reading frame (ORF) and which complement $TK^-$ strains of *Escherichia coli*, and (iv) a comparison of the amino acid sequence of the product of the BXLFI ORF with that of the HSV TK (14).

To demonstrate further that a TK enzyme can be found in both the BXLFI-expressing bacteria and the two transformed mammalian cell lines, cell extracts were prepared and assayed for TK activity (Fig. 1). Extracts from the mammalian $TK^-$ parental cell lines or from *E. coli* C600 tdK (9) showed no detectable TK activity, whereas extracts from $TK^+$ control cell lines (3T3 or L) or from *E. coli* transformed with a plasmid expressing the HSV TK were highly active. Extracts from both the 3T3 $TK^-$ cells microinjected with whole EBV (3T3 EBV) and EBV *Sal*I-B-transformed (L *Sal*I-B) cell lines and from *E. coli* transformed with plasmids expressing the EBV BXLFI ORF all contained considerable TK activity. Further evidence that the plasmid expressing the BXLFI ORF codes for a TK enzyme is shown by the detection of such activity in the products of in vitro transcription-translation assays directed by using the pUC8X plasmid (data not shown).

One characteristic of the HSV TK is the broad range of nucleoside substrates accepted by the enzyme, compared with its mammalian counterpart (7, 8), and there is some evidence that the TK obtained from EBV-infected lymphoblastoid cells has a similar wide range (10, 16, 19). We were interested in determining whether the activity found in cell extracts from EBV-transformed cell lines or from bacteria expressing the EBV TK had similar characteristics. Extracts prepared from 3T3 (EBV), 3T3 $TK^+$ mammalian cell lines, and bacteria transformed with plasmid pSK-1 (HSV TK) or pUC8X (EBV TK) were assayed for their ability to utilize the nucleosides deoxyadenosine, deoxycytidine, deoxyguanosine, and deoxyuridine and the antiviral nucleoside analogs acyclovir [9-(2-hydroxyethoxy-methyl)guanine] and cytosine arabinoside as substrates (Table 1). Extracts obtained from 3T3 $TK^+$ cell lines showed high levels of TK activity but only low levels of deoxycytidine, deoxyadenosine, deoxyguanosine, or deoxyuridine kinase activity. In addition only low activity was detected with acyclovir or cytosine arabinoside as substrates. Extracts from the 3T3 $TK^-$ cell line showed no detectable TK activity but utilized other nucleosides and nucleoside analogs to a similar extent as did the 3T3 $TK^+$ cell line (data not shown). In contrast, extracts from the 3T3 (EBV) cell line or from bacteria expressing the EBV or HSV TK showed both high levels of TK activity and relatively high levels of deoxycytidine, deoxyadenosine, deoxyguanosine, and deoxyuridine kinase activity. Finally, extracts from these systems also showed reasonable levels of acyclovir and cytosine arabinoside kinase activity.

Thus, it is apparent that the TK activity found in parental 3T3 $TK^+$ cells does not phosphorylate the nucleoside analogs acyclovir or cytosine arabinoside, whereas that found in 3T3 $TK^-$ cells transformed to express the EBV TK does. This finding is in agreement with a previous report (4) of the failure by the cellular TK to phosphorylate such nucleoside analogs and this differential phosphorylation forms the basis of the selective antiviral effect of these compounds.

In order to characterize further the TK activity found in extracts from the 3T3 (EBV) cell line and from bacteria

* Corresponding author.

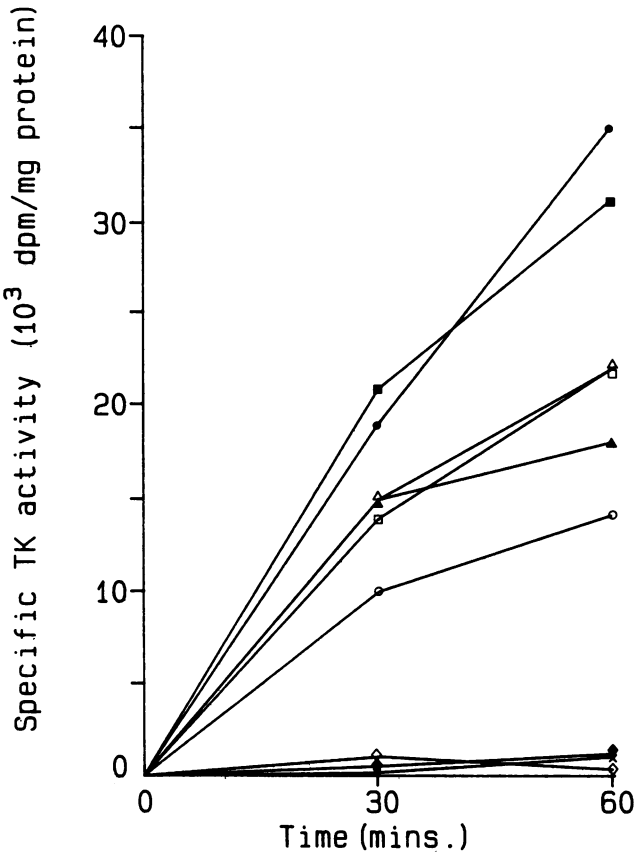


FIG. 1. TK activity in cell extracts of EBV TK expressed in transformed mammalian cell lines and in procaryotic cells. Extracts were made from mammalian cell lines LTK$^-$ (◆), 3T3 TK$^-$ (◇), 3T3 TK$^+$ (●), LTK$^+$ (■), 3T3 (EBV) (□), and L *Sal*I-B (▲) by suspension of the cells in 50 mM Tris hydrochloride (pH 8.0)–50 mM KCl–1 mM $MgCl_2$–1 mM ATP–5 mM 2-mercaptoethanol (2ME)–10 μg of phenylmethylsulfonyl fluoride (PMSF) per ml and a subsequent brief sonication. *E. coli* C600 tdK transformed with plasmid pUC8 (×), pSK-1 (△), pUC8X (○), or pUC8rX (×) was grown in minimal medium containing 30 μg each of ampicillin, glucose, and thiamine per ml to an optical density of 550 nm of 0.5. Subsequently, the bacteria were lysed in 25 mM Tris hydrochloride (pH 8.0)–50 mM glucose–10 mM EDTA–5 mM 2ME–10 μg of PMSF per ml–5 mg of lysozyme per ml for 5 min at 4°C. Extracts were then sonicated and clarified. TK assays were done in 50 mM Tris hydrochloride (pH 7.5) (at 37°C)–100 μm thymidine–5 mM $MgCl_2$–10 mM NaF–10 mM ATP–5 mM 2ME–10 μg of PMSF per ml. Thymidine was used at a specific activity of 0.4 μCi/μmol. The results were normalized for protein concentrations.

expressing the EBV TK, the ability of the ribonucleoside triphosphates ATP, CTP, GTP, and UTP to act as phosphate donors was investigated (Table 2). The extracts from the 3T3 TK$^+$ cell line or from wild-type TK$^+$ *E. coli* C600 showed TK activity when ATP was used as the phosphate donor, but considerably lower levels of TK activity were detected with CTP, GTP, or UTP as the phosphate donor. In contrast, although extracts from the 3T3 (EBV) cell line showed the highest activity with ATP as the phosphate donor, they exhibited significant TK activity with CTP, GTP, or UTP. Extracts from bacteria transformed with plasmids pUC8X or pSK-1 utilized the three alternative phosphate donors to a similar extent, but the extracts from pUC8X-transformed bacteria utilized GTP less efficiently than did the extracts from pSK-1-transformed bacteria.

TABLE 1. Utilization of nucleosides and nucleoside analogs by EBV TK expressed in transformed mammalian cells and bacteria[a]

| Nucleoside | Kinase activity (% of control)[b] | | | | |
|---|---|---|---|---|---|
| | 3T3 (EBV) | 3T3 TK$^+$ | pUC8X (EBV TK) | pUC8rX | pSK-1 (HSV TK) |
| Thymidine | 100 | 100 | 100 | 100 | 100 |
| Deoxyadenosine | 53 | 13 | 60 | 10 | 50 |
| Deoxycytidine | 36.5 | 10 | 40 | 2 | 70 |
| Deoxyguanosine | 33 | 12 | 20 | 4 | 20 |
| Deoxyuridine | 20 | 8 | 65 | 15 | 60 |
| Cytosine arabinoside | 19 | 2.5 | 10 | | 8 |
| Acyclovir | 12 | 1.3 | 4 | | 6 |

[a] The assays were performed under the conditions described in the legend to Fig. 1 except that thymidine was replaced by the indicated nucleosides at 0.4 Ci/μmol.

[b] The results are expressed as percentages of the amount of activity detected when thymidine was used as a substrate.

A number of nucleoside analogs, such as acyclovir, 9-([2-hydroxy-1-(hydroxymethyl)ethoxy]-methyl)guanine (DHPG), or 9-(1,3-dihydroxy-2-propoxy-methyl)-guanine (BVdU), have been shown to be competitive inhibitors of the HSV TK (10). These compounds have also been shown to have inhibitory action on the TK activity of other herpesviruses, including varicella-zoster virus and herpesvirus siamiri (1, 6). We report above that two of these nucleoside analogs, acyclovir and cytosine arabinoside, can be phosphorylated by the EBV TK expressed in eucaryotic or procaryotic systems. We were therefore interested in determining whether other nucleoside analogs could inhibit the action of the EBV TK. TK assays using thymidine (specific activity, 1 μCi/μm) as the substrate were performed with extracts of *E. coli* expressing either the HSV or EBV TK, in the presence of increasing amounts of the nucleoside analogs BVdU, DHPG, and acyclovir. TK activity was assayed over a 5-h period; the results shown in Fig. 2 were taken from the 4-h time point. Quite clearly both the HSV and EBV TKs were inhibited by each nucleoside analog, in the order BVdU > DHPG > ACV; however, acyclovir was apparently only marginally effective in inhibiting the EBV TK.

Our finding that BVdU and DHPG were stronger inhibitors of the EBV TK than was acyclovir reflects their relative phosphorylation by this enzyme (and also by the HSV-coded TK) (11, 18) and hence their relative efficacy in inhibiting EBV replication in transformed lymphoblastoid cell lines.

We utilized the systems derived to express the EBV TK to analyze some of the basic biochemical properties of the

TABLE 2. Activity of various nucleoside triphosphates as phosphate donors in TK reaction using EBV TK expressed in transformed mammalian cell lines and bacteria[a]

| Nucleoside triphosphate | TK activity (% of control) | | | |
|---|---|---|---|---|
| | 3T3 (EBV) | 3T3 TK$^+$ | pUC8X | pSK-1 |
| ATP | 100 | 100 | 100 | 100 |
| UTP | 73 | 9 | 80 | 94 |
| GTP | 56 | 19 | 20 | 32 |
| CTP | 51 | 16 | 62 | 70 |

[a] The assays were performed as described in the legend to Fig. 1 except that ATP was replaced by the indicated nucleoside triphosphates at 10 mM. The results are expressed as percentages of the TK specific activity when ATP was used.

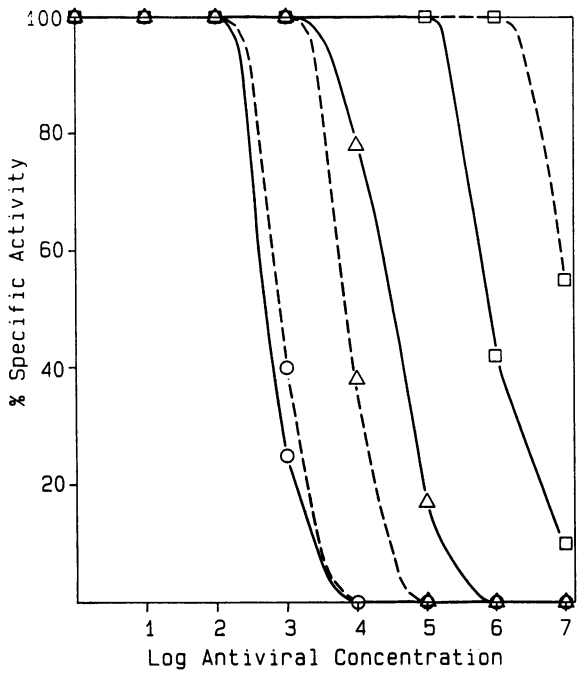


FIG. 2. Inhibition of activity of EBV and HSV TK expressed in the procaryotic system. Extracts from *E. coli* tdK containing plasmids expressing the HSV TK (——) or EBV TK (– – –) were assayed for TK activity in the presence of increasing amounts of the nucleoside analogs acyclovir (□), DHPG (△), and BVdU (○). The activity is expressed as a percentage of the TK activity observed in the absence of inhibitor. The specific activity of the thymidine used was 1 μCi/μmol, and the concentrations of the nucleoside analogs began at 0.01 μM (1 log) and increased in log steps to 5 mM (7 logs).

enzyme. The enzyme expressed in the heterologous eucaryotic and procaryotic systems appears to resemble the HSV TK in its biochemical characteristics, for example, in utilizing a broad range of substrates, including antiviral nucleoside analogs. However, a direct comparison between the characteristics of the EBV enzyme expressed in eucaryotic and procaryotic systems is not possible due to inherent differences such as posttranslational modifications or nucleoside pool levels between the two systems. Our results indicate that the EBV TK phosphorylated deoxycytidine at approximately 36% of the efficiency with which it phosphorylated thymidine, whereas the HSV TK had approximately 70% of the efficiency. Previous reports show that the HSV TK utilizes deoxycytidine at approximately 50% of the efficiency with which it utilizes thymidine (7, 8). The low activity of deoxycytidine kinase activity detected for the EBV TK may be reflected in the relative inefficiency with which the antiviral nucleoside analog acyclovir is utilized by the EBV TK (results presented here) and in its relatively poor efficiency in inhibiting EBV replication in vivo (12). As suggested by Honess et al. (6), in many herpesviruses the ability to phosphorylate deoxycytidine correlates with the ability to accept acyclovir as a substrate. Our results for inhibition of the EBV TK by nucleoside analogs show that acyclovir is a poor substrate for the EBV TK and that the analogs BVdU and DHPG are more inhibitory for the enzyme.

More detailed biochemical analysis of the EBV TK expressed in these systems will depend upon obtaining large amounts of pure enzyme. The utilization of the antiviral nucleoside analogs cytosine arabinoside, acyclovir, DHPG, and BVdU as substrates by the EBV TK is demonstrated by the phosphorylation of both cytosine arabinoside and acyclovir by the EBV TK expressed in both mammalian and procaryotic systems. In addition, the utilization of acyclovir, DHPG, and BVdU by the EBV TK can be inferred from their ability to inhibit the EBV TK expressed in the procaryotic system. These results are in agreement with previous reports which show that EBV TK expressed in infected lymphoblastoid cell lines can phosphorylate antiviral nucleoside analogs such as acyclovir and cytosine arabinoside and can be inhibited by compounds such as DHPG and BVdU (11–13, 15, 16).

Finally, we suggest that the inhibition by nucleoside analogs of the EBV TK expressed in a procaryotic system may prove to be a useful secondary screen for the efficacy of novel antiviral agents.

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## LITERATURE CITED

1. **Cheng, Y.-C., T. Y. Tso, T. Hackstadt, and L. P. Mallavia.** 1979. Induction of thymidine kinase and DNase in varicella-zoster virus-infected cells and kinetic properties of the virus-induced thymidine kinase. J. Virol. **31**:172–178.
2. **Colby, B. M., P. A. Furman, J. E. Shaw, G. B. Elion, and J. S. Pagano.** 1981. Phosphorylation of acyclovir [9-(2-hydroxyethoxymethyl)guanine] in Epstein-Barr virus-infected lymphoblastoid cell lines. J. Virol. **38**:606–611.
3. **Doberson, M. J., M. Jerkofsky, and S. Geer.** 1976. Enzymatic basis for the selective inhibition of varicella-zoster virus by 5-halogenated analogues of deoxycytidine. J. Virol. **20**:478–486.
4. **Elion, G. B., P. A. Furman, J. A. Fyfe, P. Miranda, L. Beauchamp, and M. J. Schaeffer.** 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. USA **74**:5716–5720.
5. **Graessmann, A., M. Wolf, and G. W. Bornkaum.** 1980. Expression of Epstein-Barr virus genes in different cell types after microinjection of viral DNA. Proc. Natl. Acad. Sci. USA **77**: 433–436.
6. **Honess, R. W., P. O'Hare, and D. Young.** 1982. Comparison of thymidine kinase activities induced in cells productively infected with herpesvirus saimiri and herpes simplex virus. J. Gen. Virol. **58**:237–249.
7. **Jamieson, A. T., G. A. Gentry, and J. H. Subak-Sharpe.** 1974. Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J. Gen. Virol. **24**:465–480.
8. **Jamieson, A. T., and J. H. Subak-Sharpe.** 1974. Biochemical studies on the herpes simplex virus-specified deoxypyrimidine kinase activity. J. Gen. Virol. **24**:481–492.
9. **Kaehler, R., M. Strauss, and U. Kiessling.** 1984. A well transformable E. coli tdK strain suitable for direct rescue of tk gene plasmids from mammalian cells. Biomed. Biochim. Acta **43**: K25–K29.
10. **Larder, B. A., Y. C. Cheng, and G. Darby.** 1983. Characterization of abnormal thymidine kinase induced by drug-resistant strains of herpes simplex virus type 1. J. Gen. Virol. **64**:523–532.
11. **Lin, J.-C., D. J. Nelson, C. V. Lambe, and E. I. Choi.** 1986. Metabolic activation of (9[2-hydroxy-1-(hydroxymethyl)ethyoxyl]methyl)guanine in human lymphoid cell lines infected with Epstein-Barr virus. J. Virol. **60**:569–573.
12. **Lin, J.-C., M. C. Smith, and J. S. Pagano.** 1984. Prolonged inhibitory effect of 9(1,3-dihydroxy-2-propoxymethyl)guanine against replication of Epstein-Barr virus. J. Virol. **50**:50–55.
13. **Lin, J.-C., M. C. Smith, and J. S. Pagano.** 1985. Comparative efficacy and selectivity of some nucleoside analogs against Epstein-Barr virus. Antimicrob. Agents Chemother. **27**:971–973.
14. **Littler, E., J. Zeuthen, A. A. McBride, E. Trøst-Sørensen, K. L. Powell, J. E. Walsh-Arrand, and J. R. Arrand.** 1986. Identification of an Epstein-Barr virus-coded thymidine kinase. EMBO J.

5:1959–1966.
15. **Ooka, T., and A. Calander.** 1980. Effects of arabinofuranosylthymine on Epstein-Barr virus replication. Virology **104:** 219–223.
16. **Ooka, T., A. Calander, M. de Turenne, and J. Daillee.** 1983. Effect of arabinofuranosylthymine on the replication of Epstein-Barr virus and relationship with a new induced thymidine kinase activity. J. Virol. **46:**187–195.
17. **Roubal, J., and G. Klein.** 1981. Synthesis of thymidine kinase (TK) in Epstein-Barr virus-superinfected Raji TK-negative cells. Intervirology **15:**43–48.
18. **St. Clair, M. H., W. H. Miller, R. L. Miller, C. U. Lambe, and P. A. Furman.** 1984. Inhibition of a cellular α DNA polymerase and herpes simplex virus-induced DNA polymerases by the triphosphate of BW759U. Antimicrob. Agents Chemother. **25:** 191–194.
19. **Stinchcombe, T., and W. Clough.** 1985. Epstein-Barr virus induces a unique pyrimidine deoxynucleoside kinase activity in superinfected and virus-producer B cell lines. Biochemistry **24:** 2027–2033.