Induction of Both Thymidine and Deoxycytidine Kinase Activity by Herpes Viruses

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SUMMARY

Genetical and biochemical evidence indicates that herpes simplex virus (types 1 and 2) specifies a unique kinase activity which is able to phosphorylate both thymidine and deoxycytidine. Pseudorabies virus specifies an enzyme which has only thymidine kinase activity and so does vaccinia virus, while equine abortion virus does not induce either activity.

It is shown that the HSV specified deoxypyrimidine kinase is indispensable for virus growth in serum-starved cells but not important for virus replication in actively growing culture cells.

INTRODUCTION

Kit et al. (1963) obtained a line of L cells (LTK−) which were resistant to 5-bromodeoxyuridine (B UdR), an analogue of thymidine (TdR), and could be shown to lack thymidine kinase activity (TK). They demonstrated, by infecting these cells in the presence of B UdR with either vaccinia or herpes simplex virus (HSV), that the yield of progeny virus in both cases was severely depressed. Moreover, they isolated mutants of both viruses which could grow equally well in these LTK− cells whether or not B UdR was present. Thus their studies suggested that the virus genome of both vaccinia and herpes simplex viruses contain information to code for thymidine kinase activity (TK). Subsequent biochemical investigations with HSV and LTK− cells suggested that the thymidine kinase found after infection differs from that found normally in host cells. Similar conclusions were reached by Klemperer et al. (1967) using BHK C13 cells, who, in addition, showed that the enzyme induced after herpes virus infection was immunologically distinct from the host enzyme.

Meanwhile, Subak-Sharpe had selected a cell line derived from polyoma virus transformed BHK cells which lacked both deoxycytidine (dCK) and thymidine kinase activity (TK). The analogue used for selecting for loss of TK was B UdR and that used for selecting for loss of dCK was cytosine arabinoside (araC). It was found that dCK was produced in these cells by infection with HSV but not with vaccinia (Hay et al. 1970). AraC resistant mutants of HSV were isolated and it was concluded that the dCK was a specific HSV coded polypeptide. Herpes simplex virus is not the only virus which induces deoxycytidine kinase activity: Kara & Weil (1967) have reported an increase in this activity following polyoma virus infection of mouse kidney cells, although this increased activity appears to be the result of derepression of a host specified activity.

Hay et al. (1970) in the same series of experiments reported that the mutants selected for loss of thymidine kinase activity had simultaneously lost dCK activity, and conversely

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Baby hamster kidney cells (BHK)

\[ \text{Cloned} \]

BHK C13 cells

\[ \text{Polyoma virus transformed} \]

PyY cells

\[ 0.2 \mu g/ml \text{ thioguanine single step} \]

PyY/TG cells

\[ 0.2 \mu g/ml \text{ to } 50 \mu g/ml \text{ araC multistep} \]

PyY/TG/CAR → PyY/TG/CAR cells

\[ 5 \mu g/ml \text{ to } 1 \text{ mg/ml BUdR multistep} \]

PyY/TG/CAR/BUdR cells

Fig. 1. Selection procedure employed in the derivation of PyY/TG/CAR/BUdR cells.

Mutants selected for loss of dCK activity had also lost TK activity, which suggested that both enzyme activities were due to a common protein.

This unexpected finding prompted the present investigation and the experiments reported here are concerned with detailed genetical and biochemical investigation of HSV specified deoxycytidine and deoxythymidine kinase activities in these PyY/TG/CAR/BUdR cells.

METHODS

Cell lines. BHK C13 cells (Macpherson & Stoker, 1962) grown in Eagle’s medium containing (v/v) 10% tryptose phosphate broth and 10% calf serum (ETC) were used for all virus plaque assays. The BUdR and araC resistant cells were originally obtained in our laboratory by one of us (J. H. Subak-Sharpe) as illustrated in Fig. 1. A polyoma transformed clone of BHK C13 cells (PyY cells) was first selected for resistance to the purine analogue thioguanine (TG) in a single step by cloning cells which grew in 0.2 \( \mu g/ml \) of the analogue. These cells were then grown in increasing concentrations of araC from 0.2 \( \mu g/ml \) up to 50 \( \mu g/ml \) and the survivors cloned. The cells were then exposed to 5 \( \mu g/ml \) BUdR and the survivors subjected to increasing concentrations of BUdR up to 1 \( \text{mg/ml} \). Cell lines resistant to araC but not exposed to BUdR were cloned and stored at the end of the araC selection procedure (Fig. 1).

In acquiring resistance to araC the cells lost their ability to incorporate deoxycytidine,
Herpes virus specified deoxypyrimidine kinase

and cells subsequently selected for BUdR resistance lost their ability to incorporate thymidine as determined by autoradiography.

**Virus strains.** Herpes simplex virus type 1 strain 17 syn, strain 34 Mp (derived from the macroplaque strain of Roizman (1962)) and the Kit strain (3006), herpes simplex type 2 strain 52, pseudorabies virus Kaplan strain, vaccinia virus, Evans strain, and equine abortion virus were all adapted to grow equally well in both BHK C13 cells and in PyY/TG/CAR/BUdR cells by multiple (7 to 10) serial passages in the mutant cells. Stocks of virus were then regularly grown in BHK C13 cells.

**Production of resting cells.** BHK C13 cells were seeded at a concentration of 10⁶ cells/50 mm Petri dish in 5 ml Eagle's medium supplemented (v/v) with 10% calf serum (EC10). After incubation for 24 h at 37 °C the medium was removed, the cell sheet washed, 5 ml Eagle's medium containing (v/v) 1% calf serum (EC1) added and cells incubated for 5 to 6 days at 37 °C (Burk, 1966).

**One step growth curve.** 50 mm Petri dishes (Nunclon) containing confluent monolayers of cells were infected with virus at an input multiplicity of 10 in a total vol. of 0.2 ml. After adsorption for 1 h at 37 °C, the cell sheet was washed, to remove unadsorbed virus, and 5 ml EC10 added. (In the case of resting cells the original EC1 was replaced.) At subsequent times after infection the cell sheets were suspended in the medium and virus released by ultrasonic vibration. The liberated virus was then titrated for infectivity in BHK C13 cells (Russell, 1962).

**Isolation of mutant virus.** Wild type virus pre-adapted to PyY/TG/CAR/BUdR cells was grown in BHK C13 cells in 5 ml ETC containing 5 μg/ml of BUdR for 24 h. The resulting progeny was harvested by scraping the cells into the medium, and then releasing the virus by ultrasonic vibration. The virus from each infected Petri dish was kept separate. 0.1 ml of each extract was then used to infect 1 ml (2 x 10⁶) suspended PyY/TG/CAR/BUdR cells by shaking the virus cell mixture for 30 min at 37 °C. The infected cells were then plated in 4 ml EC10 containing either 1 mg/ml BUdR or 25 μg/ml araC. After 2 to 4 days incubation at 37 °C, single plaques were picked, one from each infected plate, into 1 ml PyY/TG/CAR/BUdR cells and grown up in the presence of the appropriate inhibitor until most of the cell sheet was destroyed. The analogue resistant progeny virus was harvested as before and stored at −70 °C. Initial stocks of mutant virus were grown up in 20 oz bottles of PyY/TG/CAR/BUdR cells in the presence of the appropriate inhibitor. Subsequent stocks of virus were grown in BHK C13 monolayers in revolving Winchester bottles.

All cell lines and virus stocks were checked for freedom from mycoplasma contamination both by staining (Fogh & Fogh, 1964) and attempted culture (Randall et al. 1965) and were found to be free.

**Drug inhibition studies.** PyY/TG/CAR/BUdR cells monolayers seeded 24 h previously at 10⁶ cells per plate were infected at an input multiplicity of 1 p.f.u./cell. After one hour’s adsorption at 37 °C excess virus was removed and 4 ml EC10 was added. When required, araC (25 μg/ml) or BUdR (1 mg/ml) was added at this time. After 24 h incubation at 37 °C the cells were scraped into the medium and the resulting suspension treated in a sonifier. The sonicate was either stored at −70 °C or titrated immediately in BHK C13 cells.

**Production of enzymes.** Revolving Winchester bottles containing a cell sheet which had just reached confluence, i.e. containing approx. 3 x 10⁶ (BHK C13), or 10⁶ (PyY/TG/CAR/BUdR) cells were infected at an input multiplicity of 10 p.f.u./cell in 20 ml of EC10 and the virus allowed to adsorb at 37 °C for 1 h. 100 ml EC10 was then added and incubation continued. Eight h after infection the cells were removed in 10 ml 1:4 trypsin versene. After all the cells had detached from the glass 1 ml of calf serum was added to neutralize the
trypsin. The cells were then pelleted, washed twice in 10 ml of ice cold saline and once in 10 mM-tris, pH 7-5, containing 5 mM-mercaptoethanol, and 5 μM-thymidine/deoxycytidine. The cells were then resuspended in 5 ml buffer and disrupted by sonicating 4 × 30 s in the Dawe sonicator. The resulting sonicate was then centrifuged at 10,000 g for 1 h. The supernatant fluid was removed and used as the crude enzyme fraction. The enzyme could be stored for long periods of time at −70 °C.

**Enzyme assays.** The enzyme assay mixture contained in a final volume of 100 μl. (a) Thymidine kinase assay. 10 μM-[3H]-thymidine, 10 μCi/μmol, 10 mM-ATP, 10 mM-MgCl₂, 0.02 M-phosphate buffer, pH 6, and 50 μl enzyme fraction. For assay of the cellular enzyme a 50 mM-tris, pH 8, buffer was used. (b) Deoxycytidine kinase assay. 10 μM-[3H]-deoxycytidine (10 μCi/μmol), 10 mM-ATP, 10 mM-MgCl₂, 50 mM-tris, pH 7, and 50 μl enzyme (assay of Mrs L. Edwards, personal communication). For assay of the cellular enzyme the tris buffer was at a pH of 7.5. 50 μl enzyme usually contained 100 μg protein (Lowry et al. 1951). After addition of the enzyme fraction, the tubes were shaken and incubated at 37 °C for 15 min except where explicitly stated. The reaction was stopped by immersing the mixtures in a boiling water bath, followed by cooling in ice.

**Separation of the reaction products.** Normally the phosphorylated compounds were separated from the nucleoside by spotting 10 μl of the reaction mixture on Whatman DE81 paper discs, which were then washed once in 4 mM-ammonium formate + 10 μM-TdR/CdR at 37 °C for 7 min, once in 4 mM-ammonium formate at 37 °C for 7 min, and once for 5 min in distilled water at room temperature. The discs were finally rinsed in absolute alcohol, dried and counted in a scintillation spectrometer using a toluene based scintillant (2.5 l toluene, 12.5 g PPO and 0.125 g POPOP).

Since the reaction mixture used in these experiments is essentially a crude one, the monophosphate will be further phosphorylated by nucleotide kinases in the enzyme preparation. Total separation of all the nucleotides formed in the reaction was achieved, using one or a combination of the following solvents. The standard solvent used to separate TdR derivatives was: butanol/glacial acetic acid/water, in a v/v ratio 2/1/1. Initially this solvent was also used to separate CdR derivatives, but the picture here is complicated by the formation of deoxyuridine derivatives by contaminating dCMP deaminase present in the enzyme preparation. A pyridine/isopropanol/glacial acetic acid/water solvent in a v/v ratio of 8/8/1/4 was used to separate CdR, UdR, dCMP, and dUMP, while the separation of dCTP and dUTP was achieved using a pyridine/methanol/glacial acetic acid/water solvent in a v/v ratio of 6/6/1/4 (Gordon, Thornburg & Werum, 1962). All chromatography was performed by mixing 20 μl of the reaction mixture with 10 μl of cold nucleotides (1 mM), applying this mixture to Whatman No. 1 paper and running in descending chromatography for 16 h. After the paper had been air dried those run in the butanol solvent could be observed directly under u.v. light and the spots formed by the unlabelled controls added to the reaction mixture located. Chromatograms run in either of the pyridine solvents had to be washed extensively in CHCl₃ before the spots could be located. After location of the spots the chromatograms were cut into 1 cm strips, perpendicular to the direction of the solvent, and these strips counted as before.

**RESULTS**

**Drug resistance of wild type viruses**

The replication of herpes simplex virus type 1, is inhibited by both BUdR and araC, in PyY/TG/CAR/BUdR cells which themselves grow in the presence of and are fully resistant to both analogues. The 24 h yield of strain 17 syn virus is reduced to 0.8 % and 5 % of the
Herpes virus specified deoxypyrimidine kinase

Table I. Inhibition of herpes virus by BUdR and araC growing in PyY/TG/CAR/BUdR cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Control yield p.f.u./ml</th>
<th>Yield in the presence of BUdR 1 mg/ml</th>
<th>Yield in the presence of araC 25 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Herpes simplex:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>type I, strain 17 syn</td>
<td>8.2 × 10⁶</td>
<td>6.5 × 10⁶ (6.8 %)</td>
<td>4.1 × 10⁶ (5.5 %)</td>
</tr>
<tr>
<td>type I, strain 34 Mp</td>
<td>3 × 10⁶</td>
<td>3 × 10⁶ (0.1 %)</td>
<td>1.8 × 10⁶ (0.6 %)</td>
</tr>
<tr>
<td>type I, strain Kit</td>
<td>2 × 10⁶</td>
<td>1 × 10⁶ (0.5 %)</td>
<td>1.2 × 10⁶ (6 %)</td>
</tr>
<tr>
<td>type 2, strain HSG52</td>
<td>9.5 × 10⁶</td>
<td>8.4 × 10⁶ (0.9 %)</td>
<td>4 × 10⁵ (5 %)</td>
</tr>
<tr>
<td>(b) Pseudorabies</td>
<td>4 × 10⁷</td>
<td>8 × 10⁷ (2 %)</td>
<td>3.9 × 10⁷ (98 %)</td>
</tr>
<tr>
<td>Equine abortion†</td>
<td>2.3 × 10⁴</td>
<td>2.4 × 10⁴ (105 %)</td>
<td>2.3 × 10⁷ (100 %)</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>2.5 × 10⁶</td>
<td>6 × 10⁶ (4 %)</td>
<td>1.5 × 10⁷ (100 %)</td>
</tr>
</tbody>
</table>

* Figures in parentheses give the yield as a percentage of the control yield.
† A second strain of equine abortion virus obtained from Professor P. Wildy gave similar results.

| Table 2. Yield of HSV-1, strain 17 syn, in PyY/TG/CAR/BUdR cells after 24 h at 37 °C |
|---------------------------------|---------------------------------|---------------------------------|
| Addition                       | Titre p.f.u./ml                 | % of control                    |
| None                           | 6.4 × 10⁶                      | 100                             |
| araC* (25 µg/ml)               | 2.5 × 10⁷                      | 4                               |
| araC (25 µg/ml + 10⁻⁴ M-CdR)   | 3.9 × 10⁷                      | 65                              |
| araC (25 µg/ml + at 4 h p.i. 10⁻⁴ M-CdR) | 3.6 × 10⁷ | 6                               |
| BUdR† (1 mg/ml)                | 6 × 10⁶                       | 1                               |
| BUdR + 5 × 10⁻⁴ M-TdR         | 4.4 × 10⁶                     | 70                              |
| BUdR + at 4 h p.i. 5 × 10⁻⁴ M-TdR | 1.6 × 10⁶ | 25                              |

* 25 µg/ml = 10⁻⁴ m-araC.
† 1 mg/ml = 3 × 10⁻³ m-BUdR.

control in the presence of BUdR and araC, respectively (Table 1a). Two other type 1 virus strains (34 Mp and Kit) were tested. They were found to be sensitive to both analogues, both being inhibited to the same extent as strain 17 syn by BUdR, while strain 34 Mp showed five to tenfold greater sensitivity to araC than either of the other strains. Herpes simplex type 2 shows a similar pattern of sensitivity to BUdR as the three type 1 strains and to araC as strains 17 syn and Kit (Table 1a). The titres obtained after 24 h growth of HSV-2 in the presence of BUdR and araC were 0.9 % and 5 % respectively of the control value.

The inhibition of virus replication caused by araC can be prevented by the simultaneous addition of approximately equimolar amounts of deoxycytidine, but cannot be reversed by addition of deoxycytidine four hours after the araC. However, the inhibition of virus replication by BUdR can not only be prevented by simultaneous addition of thymidine, but is at least partially reversed by the addition of thymidine four hours after infection (Table 2).

When grown in PyY/TG/CAR/BUdR cells, pseudorabies virus and equine abortion virus, two other members of the herpes group, exhibit patterns of response to BUdR and araC which differ from that of the herpes simplex viruses. Pseudorabies is sensitive to BUdR but totally resistant to araC, while equine abortion virus is resistant to both analogues (Table 1b). Vaccinia virus, chosen as a control outside the herpes group, is sensitive to BUdR but not to araC (Table 1b).

The resistance of pseudorabies virus to araC could reflect an insufficient concentration of the drug but this does not appear to be the explanation. At higher levels of araC the yield of pseudorabies virus is indeed slightly diminished, however, the effect is qualitatively not comparable to the effect on herpes simplex virus (Fig. 2). Increasing concentrations of
araC have little or no effect on either equine abortion, or vaccinia virus; neither does the addition of $10^{-5}$ M-5-fluorodeoxycytidine in conjunction with araC affect either virus, although on herpes simplex virus it produces a greater effect than does araC on its own.

Drug resistance of mutant viruses

Mutants of herpes simplex selected either for resistance to BUdR or to araC (as described in the Methods section) were tested for their ability to grow in the presence of either BUdR or araC. One mutant of each of the three type 1 strains and of the type 2 strain which were selected for resistance to BUdR were all found to have acquired simultaneous resistance to araC; similarly, each of the mutants selected for resistance to araC had at the same time acquired resistance to BUdR (Fig. 3).

To investigate the generality of this finding many different mutants of HSV-I strain 17 syn which had been selected only for resistance to either BUdR or to araC were tested with both analogues. In all cases, irrespective of the analogue with which they had initially been selected, the mutants had simultaneously acquired resistance to both. Altogether forty mutants have been tested. The results obtained with a representative fourteen are shown in Fig. 4.

Because all mutants of HSV selected for resistance to araC showed cross resistance to BUdR and vice versa, an attempt was made to derive mutant virus under conditions selective for resistance to araC, but against resistance to BUdR. These conditions were obtained by using PyY/TG/CAR/BUdR cells including in the medium araC, methotrexate (plus adenine as purine source), and TdR. The methotrexate makes virus DNA replication dependent on exogenous TdR by blocking dTMP synthetase. TK is required for anabolism of TdR, and therefore the production of virus progeny will depend on the ability of parental virus to code for TK. Inclusion of araC, in contrast ensures that the amount of virus progeny will be inversely proportional to the ability of parental virus to code for dCK. These conditions therefore should select for HSV which is dCK$^-$ TK$^+$. 

Fig. 2. Inhibitory effect of araC on wild type pseudorabies virus (▼ — ▼) and herpes simplex virus (□ — □) grown in PyY/TG/CAR/BUdR cells.
Herpes virus specified deoxypyrimidine kinase

Fig. 3. Effect of BUdR and araC on the yield of wild-type and mutant viruses of different strains of herpes simplex grown in PyY/TG/CAR/BUdR cells. The yield of virus in the presence of the analogue is given as a percentage of the yield obtained when the virus is grown in the absence of either analogue. □, BUdR 1 mg/ml; ■, araC 25 μg/ml.

Fig. 4. Cross resistance of araC and BUdR resistant mutants of HSV-1, strain 17 syn, grown in PyY/TG/CAR/BUdR cells. The yield of virus in the presence of the analogue is given as a percentage of the yield obtained when the virus is grown in the absence of either analogue. □, BUdR, 1 mg/ml; ■, araC, 25 μg/ml.
Fig. 5. Response of wild type (■) and araC-resistant (□) HSV-1, strain 17 syn, to rescue from thymidilate starvation by deoxythymidine. PyY/TG/CAR/BUDR cells blocked in TMP synthesis by methotrexate were infected as described at a m.o.i. of 1 with HSV-1 wild type or an araC-resistant mutant virus. At 2 h dT was added at the concentrations indicated. Cells to which no TdR was added, as well as cells not blocked by methotrexate, were controls. At 24 h the cultures were harvested and virus yield assayed in BHK C13 cells. All points were done in duplicate.

Table 3. Selection of putative dCK- TK+ mutants of herpes simplex virus type 1 wild type

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Resulting titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mutagenize† HSV-t WT, BUDR, 1 µg/ml</td>
<td>1·2 × 10⁷</td>
</tr>
<tr>
<td>2</td>
<td>Enrich‡ progeny of step 1</td>
<td>8·0 × 10⁴</td>
</tr>
<tr>
<td>3</td>
<td>Enrich progeny of step 2</td>
<td>2·8 × 10⁵</td>
</tr>
<tr>
<td>4</td>
<td>Enrich progeny of step 3</td>
<td>2·5 × 10⁶</td>
</tr>
<tr>
<td>5</td>
<td>Enrich progeny of step 4</td>
<td>6·4 × 10⁵</td>
</tr>
<tr>
<td>6</td>
<td>Enrich progeny of step 5</td>
<td>2·5 × 10⁶</td>
</tr>
<tr>
<td>7</td>
<td>Plaque purify§ progeny of step 6 into enrichment conditions (dCK- TK+; 72 h)</td>
<td>5·2 × 10⁷</td>
</tr>
<tr>
<td>8</td>
<td>Enrich progeny of step 7</td>
<td>2·6 × 10⁷</td>
</tr>
<tr>
<td>9</td>
<td>Analyse progeny of step 8 for dCK and dTK markers</td>
<td>(see Table 5)</td>
</tr>
</tbody>
</table>

* All titrations were done in BHK C13 cells, and expressed as p.f.u./ml.
† For mutagenization, BHK C13 cells (dCK+ TK+) were infected with HSV-1, strain 17 syn (dCK+ TK+) at an input multiplicity of approx. 1 p.f.u./cell. After absorption of virus (30'), medium containing BUDR at a concentration of 1 µg/ml (3 × 10⁻⁶ M) was added and the cells incubated at 37 °C for 24 h. After incubation, the monolayers were scraped, and the resulting suspension (4 ml) was sonicated, and titred in BHK C13 cells.
‡ For enrichment, 0·1 ml of the harvested virus was inoculated into dCK- TK- cells and incubated for 24 h in medium containing 10⁻⁴ M-arac, 25 µg/ml; 10⁻⁴ M-methotrexate; 5 × 10⁻³ M-adenine; 10⁻³ M-glycine; and 10⁻³ M-TdR. For each subsequent enrichment, 0·1 ml of the previous harvested infected cells was inoculated into dCK- TK- cells and the process repeated. When necessary between steps, virus was stored at −70 °C.
§ Ten plaques were picked from the titration of step 6, and inoculated immediately into enriching conditions (dCK- TK- cells, + arac, + TdR). After incubation at 37 °C for 72 h, the infected cells were harvested and the virus produced titred and enriched as described above. Although virus was obtained from each plaque picked, only one (clone 1d) was analysed further.
**Herpes virus specified deoxypyrimidine kinase**

Table 4. Analysis of putative dCK− TK+ mutant of herpes simplex virus type 1

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Marker†</th>
<th>Control virus titre‡</th>
<th>Selected virus titre§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>2·2 × 10⁸</td>
<td>2·6 × 10⁸</td>
</tr>
<tr>
<td>BUDr</td>
<td>TK</td>
<td>1·2 × 10⁸</td>
<td>2·9 × 10⁸</td>
</tr>
<tr>
<td>araC</td>
<td>dCK</td>
<td>5·1 × 10⁶</td>
<td>6·7 × 10⁷</td>
</tr>
</tbody>
</table>

* dCK− TK− cells were infected at an input multiplicity of approx. 1 p.f.u./cell; after absorption of virus, medium was added containing 3 × 10⁻³ M-BUDr, 1 mg/ml; or 1 × 10⁻⁴ M-araC 25 μg/ml; as indicated. The cells were incubated for 24 h at 37 °C, harvested, and the virus produced titred in BHK C13 cells.
† A reduction in titre with BUDr indicates TK activity; a reduction with araC indicates dCK.
‡ Titre in p.f.u./ml of HSV-1, 17 syn, wild type.
§ Titre in p.f.u./ml of the progeny of step 8, Table 4.

In Fig. 5 is shown the response of the system to the selection for TK+. Two features are clear. First, a relatively high concentration of TdR (10⁻³ M) must be used in order to rescue wild type (TK+) HSV from the methotrexate block, and second, even then the selective pressure is not great; unrescued, the methotrexate does not depress virus production by quite 1 log. There is, nevertheless, a distinct advantage to having the TK+ marker; this is further seen by the relative lack of effect of TdR rescue on the araC-resistant mutant of HSV used as control. The selective pressure against dCK is somewhat stronger. In dCK+ HSV grown in dCK− cells, araC ordinarily lowers the titre by between 1 and 2 logs (Fig. 3, 4). To amplify the relatively weak selective pressure afforded by the system, virus mutagenized with BUDr was enriched for several passages in dCK− TK+ selecting conditions before plaque purification, as described in Table 3. The mutant thus isolated was analysed for the TK and dCK markers by comparing its resistance to araC and to BUDr with that of HSV-1 wild type. The results of this analysis, shown in Table 4, shows that a partial mutant was obtained, somewhat more resistant to araC and BUDr than HSV-1 wild type, but still much less so than HSV-1 mutants isolated in our standard single-selective conditions for resistance to araC or BUDr.

Mutants of pseudorabies and vaccinia virus selected for resistance to BUDr remain as resistant to araC as the wild type virus. One passage of equine abortion virus in the presence of BUdR or araC (or of pseudorabies virus in the presence of araC) has no effect on the ability of the resulting progeny to replicate in the presence of either analogue.

**Enzyme induction by wild type and mutant viruses**

Both araC and BUdR express their toxicity only as phosphorylated compounds. Therefore resistance could be expected to be due to a lack of the appropriate deoxynucleoside kinase activity. On infection of PyY/TG/CAR/BUdR cells with HSV-1 strain, 17 syn, a new thymidine kinase activity, can be demonstrated in vitro which phosphorylates thymidine to TMP. Because of the presence of other enzymes in the cell extract, TMP is further phosphorylated to TDP and TTP. The products of the in vitro reaction can be separated by partition chromatography on paper (Fig. 6). Extracts from cells infected with mutant virus selected for either BUdR or araC resistance, or from mock-infected cells show no detectable deoxycytidine kinase activity. A large amount of material formed during the
deoxycytidine kinase assay was found to migrate in a large peak near the origin (Fig. 7). On elution and re-running on a second chromatogram using a pyridine/methanol/water glacial acetic acid v/v ratio 6/6/4/1 solvent it could be separated into the nucleotides dCDP, dCTP and dUTP (Fig. 8). These nucleotides represent the expected products formed by normal cellular enzymes like CMP kinase, dCDP kinase and dCMP deaminase, which are present in the crude enzyme extract.

Wild-type viruses of the two other HSV-1 strains (34 Mp and the Kit strain), as well as the HSV-2 strain, were found also to induce both thymidine and deoxycytidine kinase activities, while all the analogue resistant mutants of these strains which we have isolated and tested, lack the ability to induce either activity irrespective of the analogue against which they were selected (Table 5).

After infection with pseudorabies virus there was an induction of thymidine kinase
activity, but no increase in deoxycytidine kinase. Equine abortion virus, on the other hand induced neither a thymidine nor a deoxycytidine kinase activity. Vaccinia virus, as expected, induced a thymidine kinase activity, but there was no evidence of any deoxycytidine kinase activity (Table 6). Both pseudorabies and vaccinia virus mutants selected for resistance to BUdR could no longer induce a thymidine kinase activity on infection of PyY/TG/CAR/BUdR cells and they remained unable to phosphorylate deoxycytidine (Table 6).

Phosphorylation of thymidine and deoxycytidine by extracts of infected PyY/TG/CAR/BUdR cells only takes place in the presence of ATP. Substitution of ATP by ADP or AMP fails to generate the phosphorylated deoxynucleotides (Table 7).
Table 5. Thymidine kinase and deoxycytidine kinase activity of HSV infected and mock-infected PyY TG CAR BUdR cells

<table>
<thead>
<tr>
<th>Infected with 100 p.f.u./cell of:</th>
<th>% Nucleoside phosphorylated*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>thymidine (TdR)</td>
</tr>
<tr>
<td>Mock-infected</td>
<td>0.6</td>
</tr>
<tr>
<td>17 syn††</td>
<td>57.2</td>
</tr>
<tr>
<td>34 Mp†</td>
<td>50.3</td>
</tr>
<tr>
<td>Kit†</td>
<td>47.4</td>
</tr>
<tr>
<td>Type 2†</td>
<td>70</td>
</tr>
<tr>
<td>17 syn, BUdR res††</td>
<td>0.1</td>
</tr>
<tr>
<td>34 Mp, BUdR res†</td>
<td>0.2</td>
</tr>
<tr>
<td>Kit, BUdR res†</td>
<td>0.8</td>
</tr>
<tr>
<td>Kit, araC res</td>
<td>0.3</td>
</tr>
<tr>
<td>Type 2, BUdR res</td>
<td>0.37</td>
</tr>
<tr>
<td>Type 2, araC res</td>
<td>0.58</td>
</tr>
</tbody>
</table>

* The reaction mixtures were incubated for 45 min 100% = approx. 25000 ct/min for both deoxy-nucleosides.
†† † signifies wild-type unselected virus.
* res signifies virus selected for resistance to the appropriate inhibitor.

Growth characteristics of wild type and mutant herpes simplex virus

Both wild type HSV and mutants selected for either BUdR or araC resistance, exhibit similar patterns of growth in exponentially growing BHK C13 cells and in PyY TG CAR BUdR cells, but only wild-type HSV grows to any appreciable extent in serum-starved (Burk, 1966) BHK C13 cells (illustrated for HSV-1 strain 17 in Fig. 9). In these three host systems the wild-type viruses grow well, although there are slight differences in the length of the growth cycle and in the actual yields. Thus BUdR- or araC-resistant HSV mutants,
**Herpes virus specified deoxypyrimidine kinase**

Table 6. Thymidine kinase and deoxycytidine kinase activity of virus-infected and mock-infected PyY/TG/CAR/BuDR cells

<table>
<thead>
<tr>
<th>Infected at an input multiplicity of 10 p.f.u./cell</th>
<th>% Nucleoside phosphorylated*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>thymidine (TdR)</td>
</tr>
<tr>
<td>Mock-infected</td>
<td>1</td>
</tr>
<tr>
<td>Vaccinia†</td>
<td>57.8</td>
</tr>
<tr>
<td>Pseudorabies‡</td>
<td>59.2</td>
</tr>
<tr>
<td>Equine abortion†</td>
<td>0.7</td>
</tr>
<tr>
<td>Vaccinia, BUdRmn†</td>
<td>0</td>
</tr>
<tr>
<td>Pseudorabies, BUdRmn‡</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* The reaction mixtures were incubated for 45 min, 100% = approx. 25,000 ct/min for both deoxy-nucleosides.
† ‡ See Table 5.

Table 7. Competence of mono- and di-nucleotides as phosphate donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>% Nucleoside phosphorylated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TdR</td>
</tr>
<tr>
<td>ATP</td>
<td>41</td>
</tr>
<tr>
<td>ADP</td>
<td>0</td>
</tr>
<tr>
<td>AMP</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Fig. 9. One step growth cycle of wild type (■■) and deoxypyrimidine kinaseless (dPyK-) mutant HSV-1 (△△) in (a) exponentially growing BHK C13 cells; (b) exponentially growing PyY/TG/CAR/BuDR cells; (c) resting (serum starved) BHK C13 cells.
although able to grow normally in non-resting cells lacking thymidine and deoxycytidine
kinases, cannot replicate in serum-starving resting cells where there is a low level of de novo
pyrimidine synthesis. This demonstrates that there are conditions which make the virus
specified deoxypyrimidine kinase gene an indispensable function for successful virus
replication.

**DISCUSSION**

Herpes simplex viruses type 1 and type 2 are inhibited by either BUdR or araC in cells
resistant to both compounds, and this inhibition has been correlated with the induction of
a thymidine and a deoxycytidine kinase activity in the infected cells. When four virus strains
were compared we found variation in susceptibility to araC. The reason for this is not
established but these differences may simply reflect diversity at the molecular level between
the enzyme proteins specified by the individual strains.

At the concentration of araC used in our experiments HSV-1 and HSV-2 were inhibited
to the same extent in PyY/TG/CAR/BUdR cells. Nutter & Rapp (1973) recently reported
that HSV-1 is more susceptible to araC than HSV-2 in rabbit kidney cells, and that at low
araC concentrations in hamster embryo fibroblasts HSV-1 and HSV-2 are equally sus-
ceptible. However, rabbit kidney cells as well as hamster embryo fibroblasts possess both
kinase activities which suggests the possibility that the reported differences are a consequence
of the host cell environment.

Within the herpes group there appears to be a wide spectrum of ability to induce these
enzyme activities: at one extreme HSV induces both; pseudorabies virus only induces
thymidine kinase; at the other extreme equine abortion virus induces neither thymidine nor
deoxycytidine kinase. This finding is certainly both interesting and unexpected: whether it
reflects different modes of natural infection or whether the explanation lies elsewhere remains
to be established.

One unrelated DNA virus, vaccinia virus, was tested and found to induce only thymidine
kinase activity after infection of cells lacking both thymidine and deoxycytidine kinase
activities.

We have demonstrated that HSV mutants selected for ability to grow in the presence of
BUdR also acquired the ability to grow in araC and conversely that mutants selected for
growth in the presence of araC simultaneously gained resistance to BUdR. This result has
been found to be true for three different type I and one type 2 strains. Moreover, 40 inde-
pendently isolated mutants of HSV-1, strain 17 syn, all coincidentally acquired resistance
to both base analogues. Our biochemical studies have established that resistance to BUdR
and araC results from simultaneous loss by the mutants of the wild-type ability to induce
thymidine and deoxycytidine kinase activities. These findings indicate that HSV not only
induces both thymidine and deoxycytidine kinase activities, but that these two functions
are genetically interrelated.

We will consider four molecular models which at first sight appear to be able to explain
the situation: (1) HSV induces two different enzymes which have at least one essential
subunit in common. (2) HSV induces two completely separate enzymes (which could be
(a) virus, and (b) host coded) by means of the same virus specified controlling element. (3)
HSV codes for one enzyme, which has separate active sites for the phosphorylation of
thymidine and deoxycytidine. (4) HSV codes for one enzyme, which has one common active
site for the phosphorylation of both substrates.

All mutants isolated from HSV-1 strain 17 syn by selection for either BUdR or araC
resistance turn out to lack the ability to induce both thymidine and deoxycytidine kinase
activities. Even experiments specifically designed to maximize the chance of isolating
Herpes virus specified deoxypyrimidine kinase

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dCK-TK\(^2\) mutants only yielded a mutant with partial loss of both functions. We are forced to the conclusion that hypotheses (1), (2a) and (3) appear rather unlikely to be correct. If hypothesis (1) operates then it is difficult to understand why all the independently isolated mutants should have their defect in the protein subunit which is common to both the thymidine and deoxycytidine kinase and not sometimes in one or other of the not shared subunits. An analogous argument applies to hypothesis (2a). As far as hypothesis (3) is concerned it is difficult to see why the occasional mutation inactivating only one of the active sites and not the other should not have been picked up. Of course it could be argued that the essential part of the individual sites, relative to the essential common part, is very much smaller and that not sufficient mutants have been checked. A further possibility involving mutational ‘hot spots’ will be discussed later.

The large number of mutants isolated which lack the ability to induce both activities, plus the fact that this constant relationship is observed in the three HSV-1 strains tested as well as in HSV-2 make it very unlikely that mutation in a controlling element, hypothesis (2a) or (2b), is the correct explanation. However, the virus gene coding for the controlling element or for the common subunit could contain a ‘hot spot’ for BUdR mutagenesis, and for this reason models (1) and (2), although they seem unlikely, cannot be totally rejected.

Thus the genetic evidence is really only fully consistent with hypothesis (4), that HSV specifies a unique deoxypyrimidine kinase, although hypotheses (1) to (3) are not completely ruled out. We have, however, obtained further biochemical evidence which strongly argues against hypotheses (1) and (2) (A. T. Jamieson & J. H. Subak-Sharpe, unpublished observations).

The question remains why thymidine and deoxycytidine kinase activities are induced at all by HSV, which seems able to replicate efficiently without them in standard tissue culture systems. Our observation that mutant virus can grow in cells which lack both these enzyme activities but cannot replicate in serum starved cells, seems highly pertinent for it suggests that HSV requires deoxypyrimidine kinase activity under conditions where the host cells’ de novo metabolism is low.

We consider it likely that the cells initially invaded in natural infections by herpes virus will have a low level of thymidilate metabolism and thus are more akin to resting than to non-resting cells. Our experiment therefore furnishes a plausible reason why HSV should code for deoxypyrimidine kinase activity.

If our finding that equine abortion virus does not code for any deoxypyrimidine kinase activity is confirmed for other strains of EAV then this suggests that since their evolutionary divergence from a common ancestor EAV and HSV have adapted to very different ecological niches even in terms of the stages in the cell cycle when these viruses are able to initiate a successful infectious cycle. Consistent with this hypothesis is the report by Lawrence (1971) that EAV only replicates in tissue culture cells which are in S phase.

It is a pleasure to acknowledge the expert technical assistance of Mrs Elsie Black, Mrs Pat Malloy, Mrs Martha MacNamara and Miss Pamela Lewis. One of us (G. A. G.) was supported in part by a gift from the Burroughs-Wellcome Foundation. A. T. J. thanks the Medical Research Council for an award for training in research methods.

REFERENCES


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