Subcellular Localization and Properties of Thymidine Kinase from Adenovirus-infected Cells

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(Accepted 24 March 1974)

SUMMARY

The principal cytosol thymidine kinase activity of African green monkey kidney cells increased approximately threefold at 21 to 29 h after infection with adenovirus type 5. Disc polyacrylamide gel electrophoresis (disc PAGE) analyses showed that the electrophoretic mobility relative to the tracking dye (Rm) of the cytosol thymidine kinase from both mock-infected and adenovirus type 5-infected cells was about 0.23. The cytosol thymidine kinase from normal cells also resembled the cytosol enzyme from infected cells with respect to phosphate donor specificity and sedimentation coefficient. Mitochondria from normal and virus-infected cells contained a cytosol-like enzyme and, in addition, a distinctive mitochondrial isozyme exhibiting an Rm of about 0.6 and a smaller sedimentation coefficient than the cytosol enzyme. The activity of the mitochondrial-specific isozyme of thymidine kinase (Rm = 0.6) was not significantly increased by virus infection. The ratio of the two thymidine kinase activities found in mitochondria also was not markedly changed by virus infection. The results suggest that adenovirus type 5 infection reactivates an inactive molecular form of cytosol thymidine kinase or derepresses the synthesis of the cytosol enzyme, but not that of the mitochondrial-specific thymidine kinase. Adenovirus infection does not alter the electrophoretic mobilities of the cytosol and mitochondrial thymidine kinases.

INTRODUCTION

Electrophoretic studies by Hatanaka, Twiddy & Gilden (1969) have suggested that a major normal cellular thymidine (dT) kinase component is converted into a virus-specified dT kinase after monkey and human cells, respectively, are abortively or productively infected by human adenoviruses. They observed that uninfected cells contained two molecular forms of dT kinase, a single major peak (90 to 95% of the recoverable activity), which migrated rapidly towards the anode, and a minor, more slowly migrating component. In adenovirus-infected cells, the electrophoretic pattern was altered in that the activity of the slowly migrating component was strikingly increased. Hence, the ratio of the fast component to the slow component changed from about 10 to 1 in uninfected cells to about 0.8 to 1 in infected cells. Kit et al. (1967b) and Kit, Nakajima & Dubbs (1970) also observed that human and simian adenovirus infections enhanced the dT kinase activity of dT kinase-positive cell lines. However, adenovirus infection did not stimulate the enzyme activity of dT kinase-deficient, mutant cells, suggesting that the stimulation of enzyme activity resulted either
from the derepression of a normal cellular dT kinase or the reactivation of an inactive molecular form of the enzyme.

In the preceding experiments, cells were disrupted so as to release the maximal dT kinase activity from all subcellular components. Recently, however, it was found that a genetically distinct mitochondrial dT kinase isozyme exists in normal cells. Furthermore, the mitochondrial isozyme persists in dT kinase-deficient human and mouse lines, despite the loss of the principal cytosol dT kinase from the mutant cells (Berk & Clayton, 1973; Kit, Leung & Trkula, 1973a, b; Kit & Leung, 1974a). The mitochondrial dT kinase exhibits a greater electrophoretic mobility than the cytosol dT kinase and also differs from it in isoelectric point, sedimentation coefficient, pH-optimum, Michaelis constant, ribonucleoside 5'-triphosphate donor specificity, and sensitivity to inhibition by dCTP.

Cytosol dT kinase activity is markedly reduced in stationary phase cultures, such as those commonly used to demonstrate enzyme stimulation after adenovirus-infection. A corresponding diminution of mitochondrial dT kinase activity does not occur. It therefore seemed possible that the altered electrophoretic pattern observed by Hatanaka et al. (1969) after adenovirus infection resulted from a selective enhancement of the cytosol dT kinase activity, rather than from the conversion of a fast migrating molecular form to a slow migrating form. To investigate this hypothesis, the subcellular distribution of dT kinase in adenovirus-infected cells has now been studied. Cytosol and mitochondrial dT kinases from mock-infected and from adenovirus-infected cells have also been characterized with respect to disc polyacrylamide gel electrophoresis mobilities, sedimentation coefficients, and phosphate donor specificities. The data to be presented demonstrate that the cytosol is the site of the principal dT kinase increase in infected cell cultures and that the cytosol enzyme from infected cells is indistinguishable from the cytosol dT kinase from mock-infected cells with respect to the properties analysed.

METHODS

Cell cultures and virus. CV-1, an established line of African green monkey kidney cells, was subcultured weekly in Eagle's minimal essential medium, APMEM (Auto Pow, Flow Laboratories, Rockville, Maryland), supplemented with 10% calf serum. Human KB cells were grown in the same medium but subcultured at 3 to 5 day intervals. Human adenovirus type 5 (strain adenoid 75) was obtained from the American Type Culture Collection. Virus stocks were prepared in KB cells and titrated by plaque assay on KB monolayers (Kit et al. 1970).

Infection of cells for induction of dT kinase activity. Stationary phase monkey kidney cultures abortively infected with adenovirus type 5 were used for the induction of dT kinase activity. Thymidine kinase activity is minimal in uninfected stationary phase cultures so that virus-induced enzyme increases can be measured against a low base line. Monkey kidney cells, rather than human KB cells, were selected for study because: (1) Hatanaka et al. (1969) demonstrated that dT kinase is induced after either abortive infection of monkey cells or productive infection of human cells; and (2) CV-1 monkey cell cultures can be maintained more easily in the stationary phase than KB cells.

To obtain stationary phase cultures for infection, 8 oz prescription bottles were inoculated with 10^6 CV-1 cells and the cells were incubated at 37.5 °C for 9 days. The culture medium was changed on the sixth and eighth days. The stationary phase cultures were then infected with human adenovirus type 5 at an input multiplicity of 60 to 90 p.f.u./cell. After a 1 h adsorption period at 37.5 °C, the virus inoculum was removed, fresh medium added, and the cultures were further incubated at 37.5 °C. Mock-infected cultures were similarly
Thymidine kinase of adenovirus-infected cells

Thymidine kinase of adenovirus-infected cells

Preparation of cell fractions and assay of dT kinase activity. For the preparation of cytosol and mitochondrial fractions, trypsinized cells were washed with Hanks' balanced salt solution and RSB (0.01 M-KCl, 0.0015 M-MgCl₂, 0.01 M-tris-HCl buffer, pH 7.4, at 25 °C), resuspended in RSB and allowed to swell for 10 min at 4 °C. Thymidine and epsilon aminocaproic acid (EACA), a protease inhibitor, were added to give concentrations of 0.2 mM and 0.05 M, respectively. The cells were disrupted with a Dounce homogenizer and cytosol and mitochondrial fractions were prepared (Kit et al. 1973a, 1974). The 'cytosol' fraction represents the high speed supernatant fraction obtained by centrifuging post-mitochondrial supernatant fractions at 105000 g for 1 h at 4 °C (Kit et al. 1973b).

To purify mitochondrial fractions, mitochondrial pellets were washed twice with TENS buffer (0.01 M-tris-HCl buffer, pH 7.4, at 25 °C; 0.001 M-EDTA; 0.01 M-NaCl; 0.25 M-sucrose), resuspended in 1 ml of TENS buffer and layered over a discontinuous sucrose gradient. The sucrose gradient was prepared by adding to 50 ml by 3½ inch cellulose nitrate sedimentation tubes, 5 ml of 1.5 M-sucrose and 5 ml of 1.0 M-sucrose, both in 0.05 M-tris-HCl buffer, pH 7.4, at 25 °C, 0.05 M-EACA, 0.2 mM-dT, 0.005 M-MgCl₂, and 0.01 M-KCl. The tubes were centrifuged at 4 °C for 1 h at 76 400 g in the Spinco L2 centrifuge, number SW41 Ti rotor. The mitochondria formed a discrete visible band and were collected from the interphase between the 1.0 M- and 1.5 M-sucrose solutions. The mitochondria were resuspended in TENS buffer and pelleted by sedimentation at 9500 g for 10 min. The pellets were washed with TENS buffer and enzyme buffer (0.15 M-KCl, 0.003 M-2-mercaptoethanol, 0.01 M-tris-HCl, pH 8.0, at 25 °C), resuspended in 1 to 3 ml of enzyme buffer containing 0.05 M-EACA, 0.2 mM-dT, and 0.5 % (v/v) Nonidet P40 (Shell Chemical Co., New York, N.Y.), frozen and thawed twice, sonicated for 3 min at 10 kc, and centrifuged at 105000 g for 1 h at 4 °C.

The mitochondrial and cytosol supernatant fluids were either analysed immediately or they were stored at −20 °C. To protect dT kinase activity during storage, glycerol was added to a final concentration of 10 % (v/v). Portions of the supernates were analysed for protein content and for dT kinase activity as described previously (Kit et al. 1966).

Disc PAGE analyses. Disc PAGE analyses were performed in 5 % acrylamide gels at pH 8.6 (25 °C) with dT (0.2 mM) and ATP or UTP (2.5 mM) in the upper buffer solution (Kit et al. 1973a, b, 1974), except that dT and ATP or UTP were also included in the polymerization mixture when preparing the separating and stacking gels. After completion of the disc PAGE run, the gels were sliced with a razor blade into 1 mm sections and then incubated with shaking for 1 h at 38 °C in small vials (15 x 45 mm) containing 150 μl of dT kinase reaction mixture.

The dT kinase reaction mixture contained the following constituents at the indicated final concentrations: 0.07 M-KCl; 1.3 mM-2-mercaptoethanol; 8 mM-MgCl₂; 12 mM-ATP or UTP; 4.8 mM-potassium phosphoenolpyruvate; 1.5 μg rabbit muscle pyruvate kinase (150 units/mg) (Boehringer Mannheim Corp., N.Y.); 4.5 μcuries [3H]-thymidine (50-7 Ci/mmol; New England Nuclear Corp., Boston, Mass.); and 0.1 M-tris-HCl buffer, pH 8.0, at 25 °C.

The reaction was terminated by the addition of 25 μl of 50 % trichloroacetic acid (w/v) and 20 μl samples were chromatographed on Whatman DE-81 paper to separate the nucleoside acceptor, [3H]-dT, from the product, [3H]-dTMP. To determine the amount of [3H]-dTMP formed in the reaction, the eluted spots were counted in a Packard Tri-Carb liquid scintillation spectrometer.
Enhancement of thymidine kinase activity after infection of stationary phase CV-1 cell cultures (159 x 10^6 cells/culture) with human adenovirus type 5. Thymidine kinase activity is expressed as pmol [PH]-dTMP formed in 10 min at 38 °C per μg protein. ●—●, mock-infected CV-1 cytosol enzyme; △—△, adenovirus type 5-infected CV-1 cytosol enzyme.

**Glycerol gradient sedimentation of dT kinase.** Velocity sedimentation experiments in 10 to 30% (v/v) linear glycerol gradients were employed to determine the sedimentation coefficients of dT kinase. Sedimentation was carried out in cellulose nitrate tubes containing 11 ml of 10 to 30% (v/v) glycerol in 0.15 M-KCl, 10 mM-2-mercaptoethanol, 0.2 M-dT, 0.25 mM-MgCl₂, 2.5 mM-ATP, and 0.05 M-tris-HCl, pH 8.0 (at 25 °C) (Kit et al. 1973a, b). 1.5 ml enzyme solution was layered over the glycerol gradient and centrifuged 20 h at 4 °C and 200000 g. Enzyme solutions contained 10% glycerol, 0.2 mM-dT, 0.05 M-ε-caproic acid, 0.5% Nonidet P40, and a horse liver alcohol dehydrogenase (ADH) marker. At the end of the run, 20 drop fractions were collected from the bottom of the tubes and assayed for 1 h (cytosol) or 2 h (mitochondrial fraction) at 38 °C for dT kinase activity. The sedimentation coefficients and mol.wt. of the dT kinases relative to horse liver alcohol dehydrogenase (ADH) (1 x crystallized, Worthington Biochemical Corporation, Freehold, New Jersey) were calculated by the method of Martin & Ames (1961).

**RESULTS**

*Enhancement of dT kinase activity in adenovirus type 5-infected cells*

In previous studies, dT kinase extracts were prepared by sonic disruption of adenovirus type 5-infected human KB cells (Kit et al. 1970). It was demonstrated that the total dT kinase activity of the infected cells was enhanced two to fourfold from 16 to 46 h after infection. To learn whether cytosol dT kinase was enhanced after adenovirus type 5 infection, stationary phase cultures of CV-1 monkey kidney cells were abortively infected with the virus and cultures were harvested at various times thereafter. Fig. 1 shows that cytosol dT kinase activity was enhanced about two to fourfold between 21 and 29 h after adenovirus type 5 infection.

*Distribution of dT kinase activity in cytosol and mitochondrial cell fractions*

Having verified that cytosol dT kinase activity is enhanced 21 to 29 h after adenovirus type 5 infection of CV-1 cells, an experiment was performed in which cytosol and mito-
Thymidine kinase of adenovirus-infected cells

Table I. Thymidine (dT) kinase activities of cytosol and mitochondrial fractions from mock-infected and adenovirus type 5-infected CV-1 cells

<table>
<thead>
<tr>
<th>Days after subculture*</th>
<th>Cell fraction</th>
<th>Total dT kinase activity per culture†</th>
<th>% of total dT kinase activity</th>
<th>dT kinase activity per 10⁶ cells†</th>
<th>dT kinase activity per µg protein†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Infected</td>
<td>Control</td>
<td>Infected</td>
</tr>
<tr>
<td>4</td>
<td>Cytosol</td>
<td>2330</td>
<td>2480</td>
<td>97.3</td>
<td>91.7</td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td>55</td>
<td>223</td>
<td>2.7</td>
<td>8.3</td>
</tr>
<tr>
<td>9</td>
<td>Cytosol</td>
<td>2270</td>
<td>2210</td>
<td>92.4</td>
<td>94.7</td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td>185</td>
<td>222</td>
<td>7.6</td>
<td>5.3</td>
</tr>
<tr>
<td>10</td>
<td>Cytosol</td>
<td>4170</td>
<td>16000</td>
<td>94.7</td>
<td>98.6</td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td>235</td>
<td>224</td>
<td>5.3</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* CV-1 cells were seeded at 10⁶ cells per culture. At 4 and 9 days after seeding, respectively, the cultures contained 6.7 × 10⁶ and 15.4 × 10⁶ cells per bottle. At nine days after seeding, experimental cultures were infected with adenovirus type 5. Cultures were harvested 24 h later. Cell counts at the time of harvest were 14.7 × 10⁶ and 14.9 × 10⁶ for mock-infected and adenovirus-infected cultures, respectively.

† pmol [3H]-dTMP formed in 10 min at 38 °C.

**Disc PAGE analyses of dT kinase activities**

To learn whether the electrophoretic properties of dT kinases in adenovirus type 5-infected CV-1 cells were similar to or different from those in uninfected cells, disc PAGE analyses were performed on cytosol and mitochondrial fractions. Fig. 2(a) shows that the mobility relative to the tracking dye (Rm) of the cytosol dT kinase from mock-infected mitochondrial extracts were prepared at 2 h and at 24 h after infection of stationary phase cultures. In addition, cytosol and mitochondrial dT kinases were analysed in rapidly growing, 4-day-old cultures of uninfected CV-1 cells.

Previous studies had demonstrated that dT kinase activity is high during the accelerated growth phase and declines in stationary phase cells (Kit, Dubbs & Frearson, 1965; Kit, 1967). Table I shows that the decrease in dT kinase activity of stationary phase cultures takes place in the cytosol and not the mitochondrial fraction. Thus, the dT kinase activity per µg protein (or per 10⁶ cells) of 9-day cultures was less than half that of 4-day cultures. Cytosol dT kinase accounted for about 97% of the total enzyme activity of 4-day-old cultures, but for only 92% of the total activity of 9-day-old cultures. The total cytosol dT kinase activity per culture was about the same at 9 days as at 4 days, despite the fact that the cells per culture increased from 6.7 × 10⁶ at 4 days to 15.4 × 10⁶ at 9 days after subculture. In contrast, the total mitochondrial dT kinase activity per culture was 3 to 4 times higher at 9 days than at 4 days, paralleling the increase in cell number.

In mock-infected cultures, the medium was changed at the time of infection and a moderate stimulation of cytosol and mitochondrial dT kinase activity was observed 24 h later. Table I shows that in cultures infected for 24 h with adenovirus type 5, the cytosol dT kinase activity was 3 to 4 times greater than that of mock-infected cultures. On the other hand, mitochondrial dT kinase activity was not increased by adenovirus type 5 infection. Hence, the cytosol dT kinase of adenovirus-infected cultures accounted for nearly 99% of the total dT kinase activity. The experiments demonstrate that the major site of dT kinase enhancement in adenovirus-infected cultures was in the cytosol and that the distribution of dT kinase activity in the subcellular fractions of the infected cultures resembled that of rapidly growing 4-day-old uninfected cultures.
Fig. 2. Disc PAGE analyses of dT kinase activities from (a) cytosol fractions from mock-infected (■—■) and from adenovirus type 5-infected CV-1 cells (○—○) (68 μg protein) and (b) mitochondrial fractions from mock-infected (■—■) and adenovirus type 5-infected CV-1 cells (■—■) (350 μg protein). Disc PAGE was carried out with ATP in the upper buffer solution except for the experiment shown by the line (△—△). In the latter experiment, extracts from mock-infected and adenovirus-infected cells were analysed with UTP in the upper buffer solution and with UTP as phosphate donor in the dT kinase assay. Numbers above the peaks signify electrophoretic mobility relative to the tracking dye (Rm). The total [PH]-dTMP for each of the peaks are as follows: (a) uninfected CV-1 cytosol, 43300 cts/min and adenovirus-infected CV-1 cytosol, 239000 cts/min; and (b) mock-infected mitochondria: 0.21 Rm and 0.61 Rm peaks, 35700 and 120500 cts/min, respectively; 0.21 Rm and 0.59 Rm peaks from mitochondria of adenovirus-infected cells, 55900 and 149000 cts/min, respectively. In the cytosol fractions from mock-infected and adenovirus-infected cells, calculated recoveries of dT kinase activities from the gels were 101 and 132 %, respectively. In the mitochondrial fractions from mock-infected and adenovirus-infected cells, calculated recoveries of dT kinase activities from the gels were 90 and 95 %, respectively.

In adenovirus type 5-infected cells, the total cytosol dT kinase activity recovered from the gels was about 5 times greater than that from the cytosol of uninfected cells. However, the Rm of the cytosol enzyme from infected cells (Rm = 0.22) was about the same as that of the cytosol dT kinase from mock-infected cells. Similar results have been obtained with extracts from human adenovirus type 12-infected cells (data not shown).

Fig. 2(b) shows the results of disc PAGE analyses of mitochondrial fractions from mock-infected and adenovirus-infected CV-1 cells. The purified mitochondrial extracts contained a cytosol-like dT kinase activity (Rm = 0.21) and a second enzyme activity with an Rm of about 0.60. The 0.60 Rm activity corresponds to the genetically distinct primate mitochondrial dT kinase (Kit & Leung, 1974b). In contrast to the results obtained with the cytosol extracts, the activities in the 0.60 Rm and 0.21 Rm mitochondrial dT kinase peaks
from the adenovirus type 5-infected cells were only 24% and 56% greater than the activities in the corresponding peaks from mock-infected cells. In the mitochondria from mock-infected cells, the total dT kinase activity in the 0.21 Rm peak was 30% of that in the 0.6 Rm peak. In the case of the mitochondria from adenovirus-infected cells, the dT kinase activity in the 0.21 Rm peak was 38% of that in the 0.6 Rm peak. Thus, the ratios of the activities in the two mitochondrial dT kinase peaks were not markedly changed after adenovirus infection.

We have previously shown that UTP cannot efficiently substitute for ATP as a phosphate donor in the dT kinase reaction mixture when human and murine cytosol dT kinases are assayed for enzyme activity. Furthermore, recoveries of human and murine cytosol dT kinases were exceedingly poor when ATP was omitted from the upper buffer solution during disc PAGE, or when UTP was substituted for ATP, both in the upper buffer solution and in the dT kinase reaction mixture during assay of disc PAGE gel slices (Kit et al. 1973a, b, 1974; Kit & Leung, 1974b). It is to be emphasized that this is in marked contrast to the results that are obtained with the mitochondrial-specific dT kinase isozymes and with the dT kinases induced in the cytosol fraction after herpes simplex virus types 1 and 2 infections. The mitochondrial and the herpes simplex virus-induced isozymes of dT kinase efficiently utilize UTP as phosphate donor in the dT kinase reaction and the activities of these isozymes are readily detected when UTP substitutes for ATP in the upper buffer solution during disc PAGE (Kit et al. 1973a, b, 1974).

Fig. 2(a) illustrates an experiment in which UTP was substituted for ATP in the upper buffer solution and in the assay of cytosol dT kinases from mock-infected and adenovirus type 5-infected CV-1 cells. It can be seen that no dT kinase activity was detected under these conditions. The results suggest that ATP is specifically required not only as the phosphate donor in the assay of cytosol dT kinase but also that ATP, or an alternative ribonucleoside 5'-triphosphate, is needed to protect the enzyme during disc PAGE.

To further analyse the requirements for ribonucleoside 5'-triphosphates, the disc PAGE analyses shown in Fig. 3 and 4 were carried out. In these experiments, ribonucleoside 5'-triphosphates were either omitted from the upper buffer solution or ATP or UTP was added to the upper buffer solution for disc PAGE. Following the electrophoretic runs, the gel slices were analysed with either ATP or UTP as phosphate donors in the dT kinase reaction mixture. Fig. 3 and 4 depict disc PAGE experiments on uninfected and adenovirus type 5-infected cytosol dT kinases, respectively. For the experiments of Fig. 3(d), (e), 4(d) and (e), dT kinase assays of gel slices were carried out with UTP as phosphate donor. The upper buffer solution either contained no ribonucleoside 5'-triphosphate or the upper buffer solution contained UTP. dT kinase activity was not detected in the gel slices. In contrast, when ATP was present in the upper buffer solution and also in the assay, the recoveries of cytosol dT kinases from mock-infected (Fig. 3b) and adenovirus type 5-infected (Fig. 4b) cells were 99% and 119%, respectively. When the ribonucleoside 5'-triphosphates were omitted from the upper buffer solution but ATP was employed in the assay, cytosol dT kinase activity was not detected in the gel slices. In contrast, when ATP was present in the upper buffer solution and also in the assay, the recoveries of cytosol dT kinases from mock-infected (Fig. 3b) and adenovirus type 5-infected (Fig. 4b) cells were 99% and 119%, respectively. When the ribonucleoside 5'-triphosphates were omitted from the upper buffer solution but ATP was employed in the assay, cytosol dT kinase activity was not detected in the extract from mock-infected cells (Fig. 3a) and less than 10% of the cytosol dT kinase activity was recovered after disc PAGE of the extract from the adenovirus-infected cells (Fig. 4a). It can also be seen from Fig. 3(c) and 4(c) that with ATP used in the upper buffer solution but UTP employed in the assay, only about 10% of the cytosol dT kinase activity was recovered from the gels. In this case, the gel slices probably contained some ATP which could function in the dT kinase assay. On the other hand, when UTP was used in the upper buffer solution but ATP was the phosphate donor in the assay, about half of the dT kinase activity was recovered from the gel slices (Fig. 3f).
Fig. 3. Effects of presence or absence of ribonucleoside 5'-triphosphates in upper buffer solution on the disc PAGE analyses of cytosol dT kinase (99 μg protein) from mock-infected CV-1 cells. For assay of dT kinase, gel slices were incubated with either ATP or UTP as phosphate donor in the dT kinase reaction mixture. Numbers above the peaks signify electrophoretic mobilities relative to the tracking dye (Rm). (a) no ribonucleoside 5'-triphosphate in upper buffer solution and ATP as phosphate donor in assay; (b) ATP in upper buffer solution and in assay; (c) ATP in upper buffer solution and UTP as phosphate donor in assay; (d) no ribonucleoside 5'-triphosphate in upper buffer solution and UTP as phosphate donor in assay; (e) UTP in upper buffer solution and in assay; and (f) UTP in upper buffer solution and ATP as phosphate donor in assay.

and 4f). In all instances where dT kinase activity was detected, the Rm's of the cytosol enzymes were 0.22 to 0.24. The experiments clearly show that (1) UTP can partly substitute for ATP in the upper buffer solution during disc PAGE, but UTP is not an efficient phosphate donor in the dT kinase reaction; and (2) the enzyme induced after adenovirus type 5 infection resembles the cytosol dT kinase of uninfected cells in phosphate donor specificity.

Glycerol gradient sedimentation of dT kinases

The sedimentation coefficients of cytosol dT kinases from human and mouse cell lines are equal to or greater than that of horse liver alcohol dehydrogenase (ADH; S = 5.1) (Kit et al. 1973a, b). Fig. 5(a) and (b) and Table 2 show that the sedimentation coefficients of the cytosol dT kinases from uninfected and adenovirus type 5-infected CV-1 cells are also very similar to that of ADH.
Fig. 4. Effects of presence or absence of ribonucleoside 5'-triphosphate in upper buffer solution on the disc PAGE analysis of cytosol dT kinase (91 μg protein) from CV-1 cells infected for 24 h with adenovirus type 5. For assay of dT kinase, gel slices were incubated with either ATP or UTP as phosphate donor in the dT kinase reaction mixture. Numbers above the peaks signify electrophoretic mobilities relative to the tracking dye (Rm). (a) no ribonucleoside 5'-triphosphate in upper buffer solution and ATP as phosphate donor in assay; (b) ATP in upper buffer solution and in assay; (c) ATP in upper buffer solution and UTP as phosphate donor in assay; (d) no ribonucleoside 5'-triphosphate in upper buffer solution and UTP as phosphate donor in assay; (e) UTP in upper buffer solution and in assay; (f) UTP in upper buffer solution and ATP as phosphate donor in assay.

Fig. 5. Sedimentation in linear 10 to 30 % (v/v) glycerol gradients of dT kinases from (a) CV-1 cytosol (1540 μg protein); (b) cytosol from adenovirus type 5-infected cells harvested 29 h after infection (1150 μg protein); and (c) CV-1 mitochondria (430 μg protein) (Δ—Δ). Recoveries of dT kinase activities from the gradients were 97, 99 and 71 %, respectively, for enzyme preparations shown in (a), (b) and (c). A portion of the fractions was also analysed spectrophotometrically for ADH activity (O—O).
Table 2. Sedimentation coefficients in 10 to 30% glycerol gradients and estimated mol. wt. of dT kinases from mock-infected and adenovirus type 5-infected monkey kidney (CV-I) cells

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Fraction</th>
<th>S*</th>
<th>Mol. wt.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV-I</td>
<td>Cytosol</td>
<td>5.04 ± 0.09 (10)†</td>
<td>81,300</td>
</tr>
<tr>
<td>Ad 5-infected CV-I</td>
<td>Cytosol</td>
<td>5.16 ± 0.05 (5)</td>
<td>84,200</td>
</tr>
<tr>
<td>CV-I</td>
<td>Mitochondria</td>
<td>4.64 ± 0.06 (5)</td>
<td>71,900</td>
</tr>
<tr>
<td>Ad 5-infected CV-I§</td>
<td>Mitochondria</td>
<td>4.60 ± 0.04 (4)</td>
<td>70,900</td>
</tr>
</tbody>
</table>

* Relative to horse liver alcohol dehydrogenase (S = 5.1 and mol. wt. = 83,000).
† Arithmetic mean ± s.e. mean. Numbers in parentheses signify number of determinations.
§ Enzyme extracts were prepared 21 to 29 h after infection.
§§ Enzyme extracts were prepared 24 h after infection.

The sedimentation coefficients of human and mouse mitochondrial dT kinases are consistently smaller than that of ADH (Kit et al. 1973a, b). Similarly, the sedimentation co-efficients of mitochondrial dT kinases from normal and adenovirus type 5-infected monkey (CV-I) cells are smaller than that of ADH (Fig. 5c, Table 2).

Assuming that the enzymes are globular proteins and that the partial specific volumes are the same as that of ADH, the mol. wt. can be estimated by the method of Martin & Ames (1961). The estimated mol. wt. of cytosol dT kinases from normal and adenovirus type 5-infected CV-I cells were about 81,000 to 84,000. These values agree with previous mol. wt. estimates of monkey dT kinase (80,000 to 100,000) made by Sephadex gel chromatography (Kit et al. 1967a). The estimated mol. wt. of the mitochondrial dT kinases from uninfected and adenovirus type 5-infected cells were about 71,000 to 72,000 (Table 2).

**DISCUSSION**

Human and monkey cell lines contain a major dT kinase molecular form located in the cytosol fraction. This cytosol dT kinase exhibits a disc PAGE mobility of about 0.23 and an isoelectric point of about 9.5 (Kit et al. 1973b, 1974; Kit & Leung, 1974a, b). The sp, act. of the cytosol dT kinase is high in rapidly growing cultures but decreases in stationary phase cultures. In addition, human and monkey cell lines contain a genetically distinct mitochondrial isozyme with a disc PAGE mobility of about 0.6 and an isoelectric point of about 6.0. In stationary phase cultures, the activity of the mitochondrial dT kinase increases relative to that of the cytosol dT kinase (Table 1).

The mitochondrial-specific dT kinase utilizes ATP, UTP, CTP and GTP as phosphate donors in the dT kinase reaction (Kit & Leung, 1974a, b; Kit et al. 1973b, 1974). In contrast, the cytosol dT kinase efficiently utilizes only ATP as phosphate donor. Furthermore, recovery of cytosol dT kinases from gels is very poor when ribonucleoside 5'-triphosphates are omitted from the upper buffer solution during disc PAGE, or when UTP substitutes for ATP as phosphate donor in the dT kinase reaction mixture during enzyme assay of gel slices. In contrast, recovery of the 0.6 Rm mitochondrial dT kinase is only partially reduced when disc PAGE analyses are carried out in the absence of ATP, and UTP can be substituted for ATP in the upper buffer solution and in the dT kinase assay (Kit et al. 1973b, 1974; Kit & Leung, 1974a, b). From the preceding discussion, it can be seen that when extracts containing both cytosol and mitochondrial dT kinases are analysed by disc PAGE, two dT kinase molecular forms can be detected. However, the ratios of the activities in the two molecular forms may depend upon whether rapidly growing or stationary phase cultures are analysed and upon the conditions used during disc PAGE. The mitochondrial dT kinase...
Thymidine kinase of adenovirus-infected cells

activity would be more prominent relative to the cytosol dT kinase activity in extracts from stationary phase cultures than in those from exponentially growing cells. Also, if disc PAGE analyses are carried out without a ribonucleoside 5'-triphosphate in the upper buffer solution, poor recovery of the cytosol dT kinase activity from the gels may result in an artifactual high ratio of mitochondrial to cytosol dT kinase activity.

In the disc PAGE experiments carried out by Hatanaka et al. (1969), two dT kinase molecular forms were observed. Comparison of their Fig. 1 with Fig. 2 to 4 of the present study suggests that their anodal molecular form corresponds to the mitochondrial-specific isoform and that their slowly migrating molecular form corresponds to cytosol dT kinase. In the study by Hatanaka et al. (1969), it appears that disc PAGE analyses were carried out without a ribonucleoside 5'-triphosphate in the upper buffer solution. The recoveries of dT kinase activities from the gels were not reported. These factors and the use of stationary phase cultures could account for the fact that the major dT kinase molecular form observed by Hatanaka et al. (1969) in uninfected cells was the rapidly migrating (mitochondrial) dT kinase. In extracts from adenovirus-infected cells, they observed that the electrophoretic pattern was altered so that the slowly migrating (cytosol) molecular form was more prominent relative to the anodal form. The latter finding is consistent with the results described in the present study.

The present experiments have demonstrated that the cytosol is the principal site of enhancement of dT kinase activity in adenovirus type 5-infected African green monkey kidney cells. In stationary phase cultures infected with adenovirus type 5, the cytosol dT kinase specific activity was about 3 times greater than that of mock-infected cultures. When disc PAGE and glycerol gradient experiments were performed, both ATP and dT were present in the buffer solutions so as to protect the dT kinase activities. Recovery of dT kinase activity from the gels and from the glycerol gradients was excellent (see legend to Fig. 2 and 5). The cytosol dT kinase which increased after adenovirus type 5 infection was indistinguishable from the enzyme from normal cells with respect to disc PAGE mobility, phosphate donor specificity and sedimentation coefficient.

Mitochondria from normal and virus-infected cells contained a cytosol-like dT kinase activity and, in addition, a mitochondrial-specific isozyme. Adenovirus infection did not increase appreciably the activities of the mitochondrial dT kinases and it did not alter the Rm values and sedimentation coefficients of the mitochondrial enzymes.

Previous experiments have shown that adenovirus infection enhances the dT kinase activity of cell lines which contain cytosol dT kinase, but not that of mutant cell lines which lack cytosol dT kinase activity (Kit et al. 1970). The previous experiments and the present study strongly suggest that adenovirus infection derepresses the synthesis of cytosol dT kinase or activates a pre-existing molecular form of the enzyme. The present experiments also indicate that adenovirus infection does not convert a major normal cellular dT kinase into an electrophoretically distinct, virus-specific molecular form.

This investigation was aided by grants from the Robert A. Welch Foundation (Q-163, Q-475) and by USPHS grants CA-06656-12, CA-10893-06 and 1-K6-AI 2352-11 from the National Cancer Institute and the National Institute of Allergy and Infectious Diseases. We thank Lettie Abel and Carol Dalrymple for technical assistance.
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(Received 7 January 1974)