A Genetically Distinct Thymidine Kinase in Mammalian Mitochondria

EXCLUSIVE LABELING OF MITOCHONDRIAL DEOXYRIBONUCLEIC ACID*

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SUMMARY

A thymidine kinase (EC 2.7.1.21) associated with mitochondria has been demonstrated in tissue culture both in wild type mouse cells and in cells which lack the cytoplasmic thymidine kinase. The mitochondrial thymidine kinase represents approximately 1% of the total cell thymidine kinase activity in wild type cells grown in culture and all of the activity of the mutant cells. The mitochondrial enzyme can be distinguished from the cytoplasmic enzyme on the basis of its $K_m$ for thymidine and its localization on the mitochondrial membrane. Furthermore, the presence of the enzyme in the mitochondria of the mutant cells results in exclusive labeling of mitochondrial DNA when the cells are grown in the presence of [3H]thymidine. Labeling studies in the presence and absence of methotrexate (an inhibitor of dihydrofolate reductase) demonstrate that, in the presence of exogenously added thymidine, both the product of the mitochondrial thymidine kinase and thymidine nucleotides synthesized in the cell cytoplasm are incorporated into mitochondrial DNA.

Studies of thymidine kinase (EC 2.7.1.21) in several eukaryotic systems have suggested that this enzyme may be involved in the regulation of DNA synthesis. Bollum and Potter originally demonstrated that initiation of DNA synthesis in regenerating rat liver was preceded by a dramatic increase in the level of thymidine kinase (1). Increased levels of thymidine kinase have also been demonstrated in adrenocorticotrophic hormone-stimulated hamster adrenal gland (2) and ecdysone-stimulated insect epithelium (3). A periodic increase in thymidine kinase activity during S phase of the cell cycle occurs in mammalian cells (4). Thymidine kinase levels are also increased prior to DNA synthesis in animal cells infected with DNA viruses. These include the poxviruses (5–7), adenovirus (7, 8), herpesvirus (9, 10), pseudorabies virus (11), polyoma (12–14), and SV40 (15, 16). Thus, thymidine kinase appears to be controlled in harmony with other enzymes involved in the process of DNA replication. In light of the possible regulatory role of thymidine kinase in DNA metabolism, it was interesting to note evidence which suggested the existence of a specific mitochondrial thymidine kinase in L cells. It was demonstrated that BrdUrd-resistant L cells do not incorporate BrdUrd into nuclear DNA but do incorporate this thymidine analogue into mtDNA (17). This finding suggested the existence of a mitochondrial thymidine kinase which is genetically distinct from the cellular thymidine kinase utilized in the incorporation of exogenous thymidine into nuclear DNA. This report demonstrates that there is in fact a distinct mitochondrial thymidine kinase. In addition we describe properties of the BrdUrd-resistant L cell relevant to studies involving the exclusive labeling of mtDNA.

EXPERIMENTAL PROCEDURE

Derivation and Growth of Cells—A BrdUrd-resistant mouse L cell line, designated LMTK−, was isolated by growth in increasing levels of BrdUrd as described (18). This cell line is able to grow at normal rates in the presence of 40 μg per ml of BrdUrd. No colonies arose when 10⁶ cells were plated in Medium A (Eagle’s minimal essential medium with 10% calf serum, 10⁻⁶ M L-glutamine, 10⁻⁵ M hypoxanthine, 4 × 10⁻⁷ M methotrexate, 1.6 × 10⁻⁷ M thymidine, 3 × 10⁻⁶ M glycine, 100 i.u. per ml of penicillin, and 100 μg per ml of streptomycin). Spinner-adapted cells LA9 and LMTK−, were grown in minimal essential medium containing 10% calf serum (Flow Laboratories), 10⁻³ M thymidine, 100 i.u. per ml of penicillin, and 100 μg per ml of streptomycin. Under these growth conditions both cell lines have generation times of 18 hours. Cultures were periodically examined for the presence of mycoplasma. All studies of tissue culture cells were performed on cells harvested in exponential growth, 3 to 7 × 10⁶ per ml. Stationary phase is reached under these growth conditions at 1.2 × 10⁶ per ml.

Preparation of Cell Fractions—Cells were harvested by centrifugation, suspended in 10 times their packed volume of Buffer A (0.01 M NaCl, 0.0015 M MgCl₂, 0.01 M Tris, pH 7.6), and...
allowed to swell for 5 min. They were disrupted with a sufficient amount of a mannitol-sucrose buffer (2.5 times) to give a final concentration of 0.21 M mannitol, 0.07 M sucrose, 0.005 M Tris, 0.005 M EDTA, pH 7.6. Nuclei were pelleted by centrifugation at 2 krpm in a Sorvall J2-A1 for 10 min. The "nuclear fraction" was prepared by resuspending this pellet in mannitol-sucrose buffer and centrifuging for 10 min at 2 krpm. The first 2 krpm supernatant was centrifuged at 13 krpm in a Beckman JA-20 rotor for 15 min. The supernatant of this centrifugation is designated the " soluble cytoplasmic fraction." The 13 krpm pellet was suspended in mannitol-sucrose and layered on top of discontinuous sucrose gradients consisting of 15 ml of 1.0 M sucrose, 0.005 M EDTA, 0.01 M Tris, pH 7.6, layered on top of 15 ml of 1.5 M sucrose, 0.005 M EDTA, 0.01 M Tris, pH 7.6. The gradients were centrifuged for 30 min at 22 krpm in a Beckman SW-27 rotor. Mitochondria were collected from the lower interphase and diluted 10-fold with mannitol-sucrose buffer. This suspension was spun at 15 krpm in the Beckman JA-20 rotor for 15 min. The pellet of this centrifugation is designated the " mitochondrial fraction." All procedures were carried out at 4°.

Assay for Thymidine Kinase—Thymidine kinase activity was determined by a derivation of the procedures of Breitman (19) and Bolton and Potter (1). The reaction mixture contained 5 mM ATP, 5 mM MgCl₂, 25 mM phosphate, 10 μM per ml of creatine kinase (Sigma Chemical Co.), 0.1 mM dithiothreitol, 80 mM Tris, pH 7.6, and 63 μM [methyl-³H]thymidine (1.6 Ci per mmole) in a total volume of 50 μl. The reaction was run at 37° for 15 min and terminated by the addition of 300 μl of cold 10 mM unlabeled thymidine-1 mm ammonium formate, pH 8.0, and chilling. Three hundred microliters of the terminated reaction mixture were slowly dripped through 24-mm DEAE-cellulose disks held in a filter block. The filters were washed three times with 5 ml of 1 mm ammonium formate, pH 8.0, and once with absolute ethanol, dried, and counted in a Beckman LS-230 scintillation counter. Under these conditions 100% of the DTMP remains bound to the filters and 99.8% of the thymidine is removed. In order to determine that the reaction product was indeed DTMP, our initial assays were terminated by the addition of 10 μl of cold 1.5 mM CCl₄COOH. Precipitated protein was removed by centrifugation, and aliquots of the supernatant were chromatographed using the following two systems: Whatman DEAE-cellulose paper eluted with 0.1 M HCl; Whatman No. 3MM paper eluted with isobutyric acid-concentrated NH₄OH-water (66:1:33). Over 95% of the reaction product co-chromatographed with 5'-dTMP in both chromatography systems. The assays of all thymidine kinase preparations described were linear with time for up to 30 min. One unit of thymidine kinase activity is defined as the amount of activity required to phosphorylate 1 pmole of thymidine during a 15-min incubation period under the conditions described above. The conversion of counts per min to picomoles of DTMP is based on counts of the substrate [³H]thymidine under conditions and geometry identical with that used in counting the reaction product.

Partial Purification of Thymidine Kinase—Partially purified mitochondrial thymidine kinase of L929 and LMTK- cells was prepared as follows. Mitochondria were lysed by incubation in 100 times their volume of 1 mM Tris, pH 7.6, for 30 min at 37°. Membranes of the lysed mitochondria were pelleted by centrifugation for 30 min at 13 krpm in a Beckman JA-20 rotor at 4°. The pellet was suspended in 1% Triton X-100-0.01 M KCl-0.01 M Tris, pH 7.6, and centrifuged at 38 krpm in a Beckman SW-50.1 rotor for 30 min at 4°. The supernatant of this centrifugation was then fractionated with ammonium sulfate. The precipitate obtained at 0 to 30% saturation was collected as a float after centrifugation at 10 krpm in the Beckman JA-20 rotor, dissolved in 0.01 M KCl-0.01 M Tris, pH 7.6, and dialyzed against this buffer. The specific activity was increased 10 to 15 times over that of the mitochondrial lysate with a recovery of 70 to 90%. The activity was stable for at least 1 month when stored on ice, but freezing and thawing led to rapid loss of activity. 5'-dTMP (90 μM) was stable in the presence of aliquots of this preparation when incubated 15 min at 37° under the conditions of the thymidine kinase assay. Therefore, this preparation contained no detectable thymidylate kinase activity or 5'-dTMP phosphatase activity.

Cytoplasmic thymidine kinase was partially purified from the soluble cytoplasmic fraction by the method of Shemin (14). The procedure resulted in a 5-fold increase in specific activity over the crude soluble cytoplasmic fraction with a recovery of approximately 20%. The final isolate was dialyzed against 0.01 M KCl-0.01 M Tris, pH 7.6.

Isolation of DNA—For the isolation of mtDNA, mitochondria from 6 to 10 x 10⁸ cells were lysed and dissolved in 2 ml of 1% SDS-0.005 M EDTA-0.01 M Tris, pH 8.5. For the isolation of nuclear DNA approximately 20 μl of the nuclear fraction were suspended in this same SDS buffer. For both nuclear and mitochondrial preparations, 0.5 ml of 7 M CsCl-0.002 M EDTA, 0.01 M Tris-pH 8.5, was added to the 2 ml of SDS suspension. Tubes were chilled in ice for 10 min to precipitate CsCl and protein. The supernatant of a 10 krpm, 10-min centrifugation in a Beckman JA-20 rotor was brought to a final density of 1.55 g per ml by the addition of solid CsCl and was made to contain ethidium bromide at a final concentration of 300 μg per ml. The samples were centrifuged in cellulose nitrate tubes in an SW 50.1 rotor for 18 hours at 38 krpm at 20°. Fractions were collected by dripping. Closed circular mtDNA was isolated as a lower band in the ethidium bromide-CsCl density gradient. The gradients of nuclear DNA contained a single band of DNA. Bands were collected in approximately 200 μl. CsCl and ethidium bromide were removed by dialysis against Dowex 50 resin in 0.1 M NaCl-0.001 M EDTA-0.05 M Tris, pH 8.5. Absorbance was deter-
mined in a Gilford spectrophotometer. All samples isolated had an A₂₆₀/A₂₈₀ of 1.8 to 2.0. Concentration was calculated using the relation 1.0 O.D. unit at 260 nm equals 50 μg per ml of DNA.

Transport of Thymidine—An amount of [³H]thymidine (3.0 Ci per mmole) to yield 0.9 μCi per ml was added to cultures at 5 x 10⁸ cells per ml (final thymidine concentration 0.3 μM). Immediately after the addition of label and every 5 min thereafter two 10.0-ml samples were removed from the culture and immediately centrifuged 3 min at 2 krpm in a Sorvall GLC-1 at 4°. In each case the supernatant was decanted and the cell pellet was washed twice with 10 ml of cold minimal essential medium containing 10% cell serum and 10 μm nonradioactive thymidine. The supernatant was decanted and 0.6 ml of cold 5% CCl₄COOH was added to the pellet. The resulting precipitate was suspended and pelleted by centrifugation at 4 krpm for 10 min in the GLC-1. One half milliliter of the supernatant was added to Triton X-100 tolune base scintillation fluid and counted in a Beckman LS-230 scintillation counter. Additional washings of the cell pellet with 100 times the pellet volume of...
the washing medium above decreased the final counts by less than 5% for each washing. In order to determine the molecular species containing the 3H label, aliquots of the \( \text{C}_{12} \text{H}_{23} \text{O}_{4} \text{H} \) supernatant were neutralized by the addition of NH\(_2\)OH and chromatographed on thin layer plates using the following chromatography system: isobutyric acid-concentrated NH\(_2\)OH-water (66:1:33). Spots corresponding to thymidine, 5'-dTMP, 5'-dGDP, and 5'-dGTP were cut out, eluted with 0.10 ml of water, and counted in a Triton X-100 toluene base scintillation fluid.

**RESULTS**

Thymidine Kinase Activity in Preparations of Purified Mitochondrion—A thymidine kinase activity can be demonstrated when purified mitochondria from LA9 cells are suspended in a low ionic strength buffer (Buffer A) and added to the assay mixture described under “Experimental Procedure.” However, varying levels of activity are observed using this procedure. Maximal and reproducible levels of activity are exhibited if the mitochondria are first subjected to osmotic shock (incubation in 1 mm Tris, pH 7.6, at 37° for 30 min) or lysis in Buffer A containing 1% Triton X-100. The specific activity observed in such mitochondrial preparations is in the range of 290 to 340 units per mg of protein. This range is very much less than the specific activity observed in the cytoplasmic fractions of these cells, 11,000 to 13,000 units per mg of protein. To eliminate the possibility that the mitochondrial activity was due to contamination by the cytoplasmic enzyme, mitochondria prepared from cells lacking the cytoplasmic enzyme (LMTK-) were examined. No thymidine kinase activity was detected in the cytoplasmic fraction prepared from LMTK- cells. However, the mitochondrial fraction did exhibit thymidine kinase activity when lysed osmotically or by 1.0% Triton X-100. The activities observed were in the range 150 to 180 units per mg of protein. Concentrations of Triton X-100 of 0.5% and 2% yielded identical results.

Mitochondrial Thymidine Kinase is Associated with Membrane—When LMTK- mitochondria are lysed osmotically the released activity remains associated with the membrane. Fifty microliters of a mitochondrial osmotic lysate were centrifuged at 13 krpm in a Beckman JA-20 rotor at 4° for 15 min. The pellet of this centrifugation was suspended in 50 \( \mu \)l of Buffer A, and aliquots of both the supernatant and the resuspended pellet were assayed. The results of the assay (Table I) indicate that the thymidine kinase activity is associated with the pellet of this centrifugation. In contrast, when mitochondria lysed with 1.0% Triton X-100 were subjected to the same procedure, approximately 80% of the mitochondrial thymidine kinase activity remained in the supernatant under these centrifugation conditions (Table I). We interpret these results to indicate that the thymidine kinase activity is associated with the mitochondrial membrane and that it can be dissociated from sedimentable membrane fragments with 1% Triton X-100.

The mitochondrial thymidine kinase of LA9 cells behaves similarly. When lysed osmotically the activity is recovered predominantly in the pellet of the 13 krpm centrifugation (Table I). When lysed with 1.0% Triton X-100 the activity was recovered predominantly in the supernatant (Table I). It is possible that the activity observed in the 13 krpm supernatant of LA9 osmotically lysed mitochondria is due to contamination by the cytoplasmic activity.

**Enzyme Kinetics of Mitochondrial Activity is Distinct from That of Cytoplasmic Activity**—The partially purified thymidine kinases were used in studies of enzyme kinetics. Lineweaver-Burk plots of the inverse of reaction velocity versus the inverse of thymidine concentration revealed higher \( K_m \) values for the mitochondrial thymidine kinase activity than were observed for the soluble cytoplasmic activity. The mean and standard deviation of the \( K_m \) values of the partially purified mitochondrial thymidine kinases from six different preparations of each cell line were 13.1 ± 1.8 \( \mu \)M for LMTK- mitochondrial thymidine kinase and 12.4 ± 1.2 \( \mu \)M for LA9 mitochondrial thymidine kinase. The difference between the \( K_m \) values of the LMTK- and LA9 mitochondrial thymidine kinases is not significant. The mean and standard deviation of the \( K_m \) for five preparations of the partially purified LA9 soluble cytoplasmic thymidine kinase was 2.72 ± 0.41 \( \mu \)M. Examples of the Lineweaver-Burk plots are shown in Fig. 1.

Mitochondrial and Cytoplasmic Thymidine Kinase Exhibit Similar Thermal Stability—When LMTK- and LA9 mitochondrial preparations suspended in Buffer A containing 1% Triton

\[ K_m \text{ values were determined according to the relation } K_m = \frac{V_{max} \times \text{slope}}{K_m} \text{ in Lineweaver-Burk plots. As stated by Mahler and Cordes (20) this is true only in the special case where the magnitude of any dissociation constant for a reactant and the enzyme is unaffected by the prior attachment of any other reactant to the enzyme. We do not know that this condition holds for the reactions studied. In the more general case where these conditions do not necessarily hold, } \]

\[ V_{max} \times \text{slope} = K_m + \frac{K_m K_i}{b} \]

where \( K_m \) is the Michaelis constant for the substrate whose concentration is varied, \( K_i \) is the Michaelis constant for the substrate whose concentration is fixed, \( K_m \) is the dissociation constant of the \( K_i \) for the enzyme, and \( b \) is the concentration of the fixed substrate. In these studies, \( b \) was the Mg\(^{2+}\) ATP chelate concentration, which was maintained at 5 \( \mu \)M. Therefore, while \( V_{max} \times \text{slope} \) may not precisely equal \( K_m \) it does represent a constant which characterizes kinetic properties of the enzyme.

**Table I**

<table>
<thead>
<tr>
<th>Cell</th>
<th>Lysis</th>
<th>Fraction</th>
<th>Percentage of total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>LMTK-</td>
<td>Osmotic</td>
<td>Supernatant</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pellet</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Triton X-100</td>
<td>Supernatant</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pellet</td>
<td>29</td>
</tr>
<tr>
<td>LA9</td>
<td>Osmotic</td>
<td>Supernatant</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pellet</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Triton X-100</td>
<td>Supernatant</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pellet</td>
<td>80</td>
</tr>
</tbody>
</table>
X-100 were incubated at 45° or at 55° the thymidine kinase activity of the extracts decreased rapidly with time. The kinetics of thermal inactivation at these two temperatures was essentially similar to the kinetics of inactivation of the thymidine kinase activity in the LA9 cytoplasmic fraction.

**Fraction of Total Cellular Activity Associated with Mitochondrion**—When cells were homogenized and nuclear, cytoplasmic, and mitochondrial fractions prepared as described under “Experimental Procedure,” the activity associated with the total purified mitochondrial fraction represented on the order of 1% of the total activity associated with all fractions in the LA9 cell (Table II). In the LMTK− cell no thymidine kinase activity is observed in the cytoplasmic or nuclear fractions. Consequently, the mitochondrial fraction represented the total cellular activity. In these studies the nuclear fraction was not highly purified, and the activity associated with the nuclear fraction of the LA9 cell may represent, at least in part, contamination by the cytoplasmic activity.

**Distinct Labeling of mtDNA in LMTK− Cell** The observations above are consistent with a previous report of exclusive labeling of mtDNA when BrdUrd-resistant cells are grown in the presence of radioactively labeled thymidine (17). We have studied the basis of this phenomenon in greater detail. [3H]Thymidine (3.0 Ci per mole) was added to logarithmic cultures of LA9 and LMTK− cells to a concentration of 0.9 μCi per ml. The specific activities of closed circular mtDNA and of nuclear DNA prepared from cells labeled for various times are shown in Fig. 2. The results show that in the LMTK− cell the specific activity of nuclear DNA is less than 3% of that of the mtDNA and less than 0.05% of that of the nuclear DNA of the wild type LA9 cell.

The results in Fig. 2 also show that when the LMTK− and LA9 cells are labeled under identical conditions mtDNA isolated

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**TABLE II**

**Percentage of total cellular thymidine kinase activity associated with nuclear, soluble cytoplasmic, and mitochondrial cell fractions of LA9 and LMTK− cells**

The fractions were prepared and assayed as described under “Experimental Procedure.” Data are derived from products of determinations of activity in aliquots of each cell fraction and the total volume of each cell fraction.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Cell fraction</th>
<th>Percentage of total cellular thymidine kinase activity</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA9</td>
<td>Nuclear</td>
<td>3.2</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soluble cytoplasmic</td>
<td>95.8</td>
<td>95.8</td>
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</tr>
<tr>
<td></td>
<td>Mitochondrial</td>
<td>1.0</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>LMTK−</td>
<td>Nuclear</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Soluble cytoplasmic</td>
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<tr>
<td></td>
<td>Mitochondrial</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

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**FIG. 2. Specific activity of nuclear and mtDNA of LA9 and LMTK− cells after growth in 0.9 μCi per ml of [5-methyl-3H]thymidine (3.0 Ci per mole). Label was present in logarithmically growing cultures for the times indicated. Nuclear DNA was isolated from ethidium bromide-CsCl density gradients as described under “Experimental Procedure.” Covariently closed circular mtDNA was isolated as the lower band of ethidium bromide-CsCl density gradients as described under “Experimental Procedure.” O—O, nuclear DNA; O—O, mitochondrial DNA; A, LA9 cells; B, LMTK− cells.**
It was possible that the difference in the extent of labeling of the mtDNA in LA9 and LMTK− cells was due to a difference in the rate of transport of exogenous thymidine into intracellular thymidine pools. Therefore, we examined the uptake of thymidine by LA9 and LMTK− cells under the labeling conditions described above (Fig. 3). The LA9 cell exhibited an initial rapid uptake of thymidine which approached equilibrium after 30 min. As predicted from previous work in wild type mammalian cells (22) the predominant labeled thymine nucleotide in the LA9 cell is dTTP. In contrast, the LMTK− cell failed to concentrate thymidine, and, as expected, the labeled species which was found in these cells chromatographed as thymidine.

These findings suggest that active transport of thymidine by L cells is coupled to the cytoplasmic thymidine and thymine nucleotide kinases.) In both of these cell lines the thymidine pools were maximally labeled by the time the first samples were taken. LA9 cells transported approximately 5 pmol per 10^7 cells into the total cellular thymidine pool, and LMTK− cells transported approximately 2 pmol per 10^7 cells. Thus the 20-fold difference in the extent of labeling of the LA9 and LMTK− mtDNA could not be accounted for entirely by differences in the rate of thymidine transport or differences in the thymidine pools.

Mitochondrial Transport of Thymidine and Thymine Nucleotides—In LA9 cells extramitochondrial thymidine and thymine nucleotide pools are labeled, whereas in LMTK− cells only the extramitochondrial thymidine pools are labeled. These observations, and the fact that under identical labeling conditions LA9 mtDNA has a much higher specific activity than LMTK− mtDNA, are consistent with the following scheme. Most of the thymidine incorporated into the mtDNA of these cells is transported into the mitochondrion from extramitochondrial thymidine nucleotide pools (dTMP, dTDP, or dTTP). A fraction of the thymidine incorporated into mtDNA is acted on at the membrane by the mitochondrial thymidine kinase (Fig. 4). In the LA9 cell both transport systems yield labeled intramitochondrial dTTP, whereas in the LMTK− cell only the minor transport system yields labeled intramitochondrial dTTP. Therefore, the LMTK− mtDNA incorporates only a small fraction of the label that is incorporated by the LA9 mtDNA. In the scheme as represented in Fig. 4 and in the following discussion we assume that extramitochondrial dTTP is the thymidine nucleotide transported into the mitochondrion. However, the same arguments would hold if dTMP or dTDP were transported and then further phosphorylated within the mitochondrion.

We have tested this model by examining the effects of inhibiting de novo dTMP synthesis with the drug methotrexate, an inhibitor of dihydrofolate reductase. When cells are grown in the presence of methotrexate, tetrahydrofolate is oxidized to dihydrofolate via the thymidylate synthetase reaction and cannot be reutilized. Cells become deficient in tetrahydrofolate and therefore fail to synthesize dTMP de novo (as well as purines, glycine, and S-adenosylmethionine). LMTK− cells cannot convert thymidine to dTMP in the cytoplasm. Therefore, if methotrexate is administered to these cells de novo dTMP synthesis will be inhibited resulting in a decrease in the cytoplasmic dTTP concentration. The model predicts that if [3H]thymidine is administered under these conditions the intramitochondrial dTTP specific activity will be greater than in LMTK− cells not treated with methotrexate because the dilution by transported de novo synthesized dTTP will be diminished. Therefore, after a pulse of [3H]thymidine, methotrexate-treated cells should
The mtDNA of a 600-ml culture of LMTK- cells was prelabeled by the addition of 2-[14C]thymidine (50 mCi per mmole) to 0.01 μCi per ml when the cells were at a concentration of 1.0 × 10^6 per ml. When cells reached a concentration of 5 × 10^5 per ml they were split into two equal cultures. Methotrexate was added to one culture to 0.01 PCi per ml when the cells were at a concentration of 1.0 × 10^6 per ml. When cells reached a concentration of 5 × 10^5 per ml they were split into two equal cultures. Methotrexate was added to one culture to 0.01 PCi per ml when the cells were at a concentration of 1.0 × 10^6 per ml. Five hours later cells were harvested and mtDNA was isolated as described under "Experimental Procedure." Fractions of the ethidium bromide-CsCl equilibrium density gradients were dripped onto GF/A glass fiber filters and assayed for radioactivity (Fig. 5). Counts were normalized with respect to 14C counts per min. Methotrexate-treated cells incorporated twice as much [3H]thymidine into mitochondrial DNA as control cells (Fig. 5). As a control for the above experiment we examined the effects of methotrexate on the incorporation of [3H]thymidine into the mtDNA of LA9 cells. In these cells both extramitochondrial thymidine nucleotide pools and extramitochondrial thymidine nucleotides. TdR represents thymidine. See text for explanation.

mitochondrial thymidine kinase. Methotrexate inhibits dihydrofolate reductase. UMP, TMP, and TTP refer to the deoxy-nucleotides. These findings are consistent with the model presented in Fig. 4.

DISCUSSION

Four lines of evidence support the conclusion that there is a thymidine kinase in L cell mitochondria that is distinct from the major cellular thymidine kinase. (a) LMTK- cells lack the soluble cytoplasmic activity but continue to express the mitochondrial activity. (b) When LMTK- cells are grown in media containing [3H]thymidine, mtDNA incorporates tritium while nuclear DNA does not. (c) The mitochondrial activity and the soluble cytoplasmic activity exhibit different K_m values for thymidine. (d) When purified mitochondrial preparations are lysed without the use of detergents, the mitochondrial activity remains membrane bound. This is in contrast to the major cellular cytoplasmic activity in wild type cells.

While this work was in progress, Masui and Garren reported a membrane-bound thymidine kinase activity associated with a mitochondrial fraction of hamster adrenal gland (23). They report that 80% of the thymidine kinase activity of the untreated tissue is associated with this fraction and the remaining 20% is a soluble thymidine enzyme. We have found that approximately 80% of the cellular thymidine kinase in adult C3H mouse liver is associated with the mitochondrial fraction. These findings contrast with the observation that in LA9 cells the mitochondrial thymidine kinase represents only approximately 1% of the total cellular activity (Table II). This discrepancy is primarily due to greatly increased levels of the soluble cytoplasmic thymidine kinase in tissue culture cells compared to the cells of adult animal tissue.

The difference in the extent of labeling of the mtDNA in

A. J. Berk and D. A. Clayton, unpublished results.
LMTK− and LA9 cells can be explained by our conclusion that thymidine enters the intramitochondrial dTTP pool by two different mechanisms (Fig. 4). Most of the thymidine in the intramitochondrial dTTP pools enters by transport of extramitochondrial thymidine nucleotide. A fraction of the thymidine in the intramitochondrial dTTP pools must enter by a mechanism which employs the mitochondrial thymidine kinase. Presumably the final two phosphates are then added by activities present within the mitochondrion. Nucleoside monophosphokinase and nucleoside diphosphokinase activities have been observed in mitochondrial preparations (24). The observation that mtDNA is exclusively labeled in LMTK− cells indicates that the mitochondrial thymidine nucleotide transport system is strongly unidirectional.

LMTK− cells that have been grown for more than 16 months in the presence of 40 μg per ml of BrdUrd continue to express the mitochondrial thymidine kinase and replace approximately 10% of the thymidine in their mtDNA with BrdUd. Since BrdUrd is known to be mutagenic (25) and to cause decreased transcription rates in bacterial systems (26), one might expect that LMTK− cells grown continually in the presence of BrdUrd would exhibit evidence of the effects of this drug. Electron micrographs of thin sections of LMTK− cells grown with and without BrdUrd indicate that the drug does cause an alteration in mitochondrial morphology (17). However, these cells exhibit no obvious alterations of viability or generation time in the presence of BrdUrd. One explanation for these observations might be that the mtDNA does not code for gene products required by cells in tissue culture. This is unlikely because it is known that chloramphenicol, administered to tissue culture cells at concentrations which inhibit protein synthesis on mitochondrial ribosomes specifically, leads to cell death in a few generations (27). Functioning mitochondrial ribosomes are therefore apparently required for cell growth. Since the mtDNA contains the mitochondrial ribosomal RNA genes (28, 29) one would expect that tissue culture cells require at least the expression of these mitochondrial genes.

A more likely explanation for these phenomena is that the low level of BrdUrd labeling observed is insufficient to render required mtDNA gene products nonfunctional. The basis of BrdUrd mutagenicity is believed to be the formation of BU:G base pairs at low frequency yielding AT to GC base pair transitions (25). In order to assess the extent of mutation that would occur from a random replacement of 10% of the mtDNA thymidine with BrdUd, it is necessary to know the frequency with which this aberrant base pair occurs. Approximately 75 such base pair transitions would cause a change in the buoyant density of the mtDNA of 0.001 g cm−3. No such alteration was observed when LMTK− cells grown for long periods of time in 40 μg per ml of BrdUrd were cultured in media free of BrdUrd (17). The resolution in these gradients is approximately 0.001 g cm−3. Therefore, this low level of BrdUrd labeling has led to fewer than 75 base pair transitions. This amounts to less than five base pair transitions per 1000 base pairs in the mtDNA. Because of the degeneracy of the genetic code, 31% of these random transitions will yield no change in the primary structure of mitochondrial gene products (30). Another class of mutations (the size of this class is difficult to estimate) will yield amino acid replacements which do not affect the function of the mitochondrial gene products. Furthermore, it is known that an L cell mitochondria contains an average of 6 mtDNA molecules (31). Thus any deleterious mutations in 1 molecule might be complemented by another molecule in the same mitochondrion. The mitochondrial alterations observed when these cells are grown in BrdUrd may be the result of the decreased transcription rate of BrdUrd-labeled DNA (25). In any case, the transcription rate must be sufficiently high and the mutation rate must be sufficiently low to yield the levels of functional mitochondrial gene products required for cell viability, i.e. those gene products whose absence leads to the death of chloramphenicol-treated cells.

We have shown that LMTK− cells grown in [3H]thymidine incorporate label exclusively into mtDNA. This is because these cells lack the soluble cytoplasmic thymidine kinase but maintain a distinct mitochondrial thymidine kinase. This property of the LMTK− cell has recently been utilized in studies of the detailed mechanism of mtDNA replication (21). LMTK− and other TK− lines containing different mtDNA complexities now under study in this laboratory should prove to be very useful in elucidating the biosynthesis of mitochondrial nucleic acids. A preliminary report has been made of the thymidine kinase activity in mitochondrial preparations from several TK− and wild type cell lines and animal tissue (32).

**Note Added in Proof** After submission of this manuscript we learned of the important observation that TK− mutants excrete thymidine from their own TMP pool by nucleotidase action (H. Green, personal communication). If our L cell TK− mutants were excreting a high enough level of cold thymidine, the observed differences in specific activity of mtDNA from wild type and TK− cells could be explained by an increase in the background of cold thymidine in the TK− cell. We have tested this possibility by direct measurement of the level of thymidine present in our TK− medium and by labeling mtDNA in wild type cells grown in wild type and TK− cell conditioned media. The results indicate that a maximum of 15% of the total differential labeling of wild type and TK− mtDNA could be due to an elevated background of cold thymidine excreted by the TK− cell.

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