DISTINCTIVE PROPERTIES OF MITOCHONDRIAL THYMIDINE(dT)KINASE FROM BROMODEOXYURIDINE(dBU)-RESISTANT MOUSE LINES

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SUMMARY: dBU-resistant mouse lines lack detectable dT kinase activity in the high speed supernatant (cytosol) cell fraction. However, they contain a mitochondrial dT kinase, which sediments more slowly in glycerol gradients than the cytosol enzyme of parental mouse lines, exhibits a disc PAGE mobility relative to the tracking dye (Rm) of about 0.7-0.8, and utilizes ATP, UTP, GTP, and CTP as phosphate donors. The mitochondrial fraction of parental cells also contains this 0.7-0.8 Rm activity and, in addition, a minor dT kinase activity which migrates faster than the cytosol enzyme, but utilizes only ATP as phosphate donor. The cytosol dT kinase of parental mouse lines exhibits an Rm of about 0.2-0.3 and utilizes only ATP as phosphate donor.

INTRODUCTION: Bromodeoxyuridine (dBU)-resistant mouse [LM(TK$^-$) and mKS(BU100)] and human [HeLa(BU25)] cell lines contain significant amounts of mitochondrial thymidine (dT) kinase activity, despite the loss of the principal high speed supernatant (cytosol) enzyme from the mutant cells (1-6). The data to be presented demonstrate that the mitochondrial dT kinase from enzyme-deficient mouse cells differs from the cytosol enzyme of parental mouse lines in sedimentation coefficient, ability to utilize ribonucleoside triphosphates other than ATP as phosphate donors, and in disc polyacrylamide gel electrophoresis (disc PAGE) patterns.

MATERIALS AND METHODS: Cell lines were cultivated in Eagle's minimal essential medium (Auto Pow, Flow Laboratories, Rockville, Md.) supplemented with 10% calf serum. Except for the passage immediately preceding an experiment, the media for LM(TK$^-$) and mKS(BU100) cells contained 25μg/ml dBU. The procedures used to prepare mitochondrial and cytosol cell fractions and to assay dT kinase have been described (7). Disc PAGE analyses were performed essentially as described by Gabriel (8) (see Legend to Fig. 2). The upper buffer contained both ATP and dT, the substrates for dT kinase, and dT was also present in the gel. To determine the sedimentation coefficients, enzyme preparations were centrifuged in linear (10-30% v/v) glycerol gradients in cellulose nitrate tubes

(see Legend to Fig. 1). The sedimentation coefficients and molecular weights of the dT kinases relative to horse liver alcohol dehydrogenase (ADH) (1 x

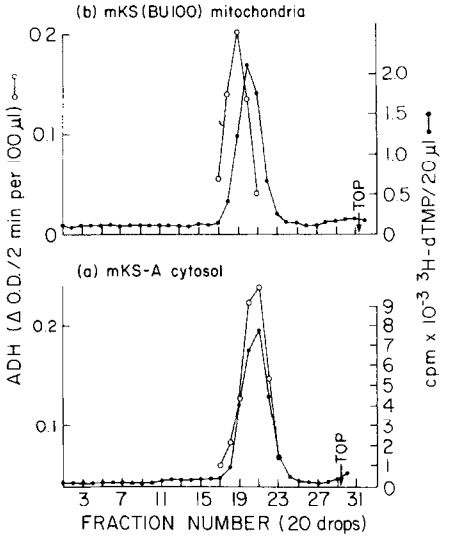

Figure 1: Centrifugation in 10-30% (v/v) glycerol gradients of dT kinase from (a) mKS-A cytosol (300 µg protein) and (b) mKS(BU100) mitochondrial (840 µg protein) cell fractions. Each tube contained 11 ml of 10-30% (v/v) glycerol in 0.15 M KCl, 10 mM 2-mercaptoethanol, 0.2 mM dT, 1.25 mM $MgCl_2$, 2.5 mM ATP, and 0.05 M Tris-HCl, pH 8.0 at 25°C. Five tenths ml of dT kinase solution was layered over the glycerol and the tubes were centrifuged in the Spinco L-2 centrifuge, SW 41 Ti rotor, for 18-20 hr at 4°C at 200,000 xg. Horse liver alcohol dehydrogenase (ADH) was added to the enzyme solution as a reference marker. Twenty drop fractions were collected from the bottom of the centrifuge tube into vials chilled at 0°C and portions were assayed for dT kinase (7). The mKS-A cytosol enzyme and the mKS(BU100) mitochondrial enzyme were incubated for 1 hr and for 2 hr, respectively, with the dT kinase reaction mixture. Ninety-one % of the cytosol and 94% of the mitochondrial dT kinase activities were recovered from the gradients. A portion of each fraction was also analyzed spectrophotometrically for ADH activity.

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crystallized, Worthington Biochemical Corp., Freehold, New Jersey) were calculated by the method of Martin and Ames (9).

RESULTS AND DISCUSSION

dT kinase activities of cytosol and mitochondrial cell fractions: Conversion of parental LM and mKS-A cells to dBU resistance is associated with the loss of the cytosol dT kinase from the mutant cells. Cytosol fractions from parental LM and mKS-A cells contain high levels of dT kinase activity; how-

ever, cytosol fractions from mutant LM(TK$^-$) and mKS(BU100) cells do not contain detectable enzyme activity (Table I). About 1% or less of the total dT

TABLE I

Thymidine (dT) Kinase Activities of Cytosol and Mitochondrial Cell Fractions*

Mouse Line	Cell Fraction	dT Kinase Activity per 10^6 cells	% of Total Cytosol plus Mitochondrial dT Kinase Activity	Specific Activity**
mKS-A	cytosol	29,000	99.7	11.2
	mitochondria	85	0.3	0.1
mKS(BU100)	cytosol	0	0	0
	mitochondria	67	100	0.1
LM	cytosol	19,000	98.9	16.2
	mitochondria	200	1.1	0.4
LM(TK$^-$)	cytosol	0	0	0
	mitochondria	140	100	0.3

* Mitochondrial extracts were centrifuged for 1 hr at 105,000 xg in the Spinco L-2 centrifuge and high speed supernant fractions assayed with ^{3}H-dU as nucleoside acceptor.

** Picomoles dUMP formed in 20 min at 38° C per μg protein.

kinase activity of parental LM and mKS-A cells is found in the mitochondrial fraction and the specific activity of the mitochondrial enzyme is very low compared to that of the cytosol dT kinase. The specific activities of the mitochondrial dT kinases of mKS(BU100) and LM(TK$^-$) cells are of the same order of magnitude as those of the mitochondrial dT kinases of mKS-A and LM cells, respectively.

Glycerol gradient centrifugation of cytosol and mitochondrial dT kinase fractions: It has consistently been found that the sedimentation coefficient of the dT kinase from cytosol fractions of dT kinase-positive human cell lines is equal to or greater than that of ADH. In contrast, the sedimentation coeffi-

cient of human mitochondrial dT kinase is less than that of ADH (7). Fig. 1 and Table II show that the sedimentation coefficient of dT kinase from the cytosol fraction of dT kinase-positive mouse lines is about 5.1-5.4 S. As in the case of human mitochondrial extracts, the sedimentation coefficient of mitochondrial dT kinase is about 4.4-4.9 S. Assuming that the enzymes are globular proteins and that the partial specific volumes are the same as that of ADH, the molecular weight of the dT kinase can be estimated by the method of Martin and Ames (9). Table II shows that the estimated molecular weight

TABLE II

Sedimentation Coefficients in 10-30% (v/v) Glycerol Gradients and Estimated Molecular Weights of dT Kinases from Cytosol and Mitochondrial Fractions of Mouse Cells

Mouse line	Cell Fraction	S*		Molecular Weight*	
mKS-A	cytosol	5.1	5.4	83,000	91,000
		5.4	5.4	91,000	91,000
	mitochondria	4.5	4.6	69,000	70,000
mKS(BU100)	mitochondria	4.7	4.6	72,000	70,000
LM	cytosol	5.3	5.1	88,000	83,000
		5.4	5.1	91,000	83,000
	mitochondria	4.5	4.4	69,000	67,000
LM(TK$^-$)	mitochondria	4.4	4.9	67,000	77,000

* Relative to horse liver alcohol dehydrogenase (S = 5.1; MW = 83,000). See legend to Fig. 1

of mitochondrial dT kinase from enzyme-deficient and from parental cell lines is slightly smaller than that of the cytosol dT kinase.

disc PAGE analyses of cytosol and mitochondrial dT kinase activities: The cytosol dT kinase of parental LM and mKS-A cells exhibits a single disc PAGE peak with a mobility relative to the tracking-dye (Rm) of about 0.2-0.3 (Fig. 2a, 3a). The cytosol dT kinase utilizes ATP, but not UTP, CTP or GTP as phosphate donors. A cytosol enzyme activity was not detected in extracts of mutant LM(TK$^-$) or mKS(BU100) cells (Fig. 2c, 3c). In contrast to the Rm of

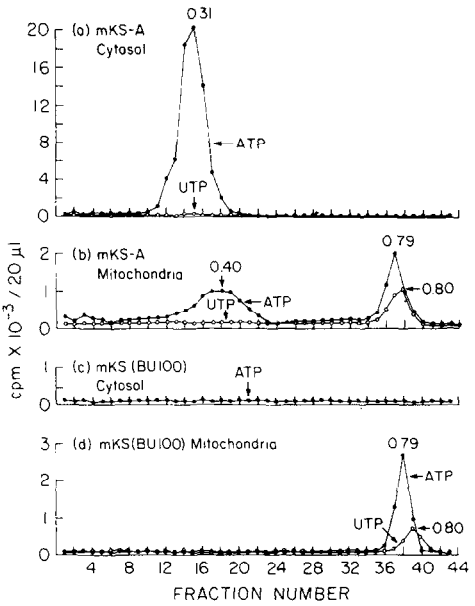

Figure 2: disc PAGE analyses of dT kinase activities of (a) mKS-A cytosol; (b) mKS-A mitochondrial; (c) mKS(BU100) cytosol; and (d) mKS(BU100) mitochondrial cell fractions.

Disc PAGE was performed in glass tubes (0.5 x 9 cm) which contained 0.3 cm of concentrating gel and 7.0 cm of separating gel. The concentrating gel consisted of the acrylamide monomer (1.88%, w/v), N, N^1-methylene bisacrylamide (BIS) (0.32% w/v), N,N,N^1,N^1-tetramethylene diamine (TEMED) (0.06%, v/v), riboflavin (0.0005%, w/v), sucrose (20%, w/v), 0.2 mM dT, and 1.25 mM $MgCl_2$, which were prepared in 0.06 M Tris-HCl buffer, pH 6.7. The separating gel consisted of the acrylamide monomer (5%, w/v), BIS (0.25%, w/v), TEMED (0.028%, w/v), riboflavin (0.0005%, w/v), 0.2 mM dT, and 1.25 mM $MgCl_2$, which were prepared in 0.38 M Tris-HCl, pH 9. The upper and lower buffer chambers contained 25 mM Tris-HCl, 0.192 M glycine, 1 mM $MgCl_2$, 0.2 mM dT, and 10 mM ME, at a final pH of 8.6 (at 25°C). The upper buffer also contained 2.5 mM ATP or UTP. Fifty microliters of enzyme sample was mixed with 5 µl of a sucrose-bromophenol blue solution [50% sucrose, 0.1% bromophenol blue (w/v), 1 mM $MgCl_2$], the mixture was layered on top of the gels, and the samples were electrophoresed at 3 ma/gel for approximately 75 min at 4°C.

At the end of the disc PAGE run, the gels were sliced in a cold room with a razor blade into 1 mm sections and the slices were immediately incubated with shaking for 1 hr (cytosol fraction) or 2 hr (mitochondrial fraction) at 38°C in small vials containing 150 µl of dT kinase reaction mixture. When UTP was used in the upper buffer solution, UTP was also substituted for ATP as phosphate donor in the dT kinase reaction mixture. The reaction was terminated by the addition of 25 µl of 50% trichloroacetic acid (w/v) and 20 µl aliquots were chromatographed on Whatman DE-81 paper to separate the nucleoside acceptor, ^{3}H-dT, from the product, ^{3}H-dTMP. The amount of ^{3}H-dTMP formed was determined using a Packard Tri-Carb liquid scintillation spectrometer.

the cytosol enzyme of parental cells, the Rm of the mitochondrial dT kinase of mutant LM(TK$^-$) and mKS(BU100) cells was about 0.8. Furthermore, the mitochondrial enzyme utilized ATP, UTP (Fig. 2d, 3d), CTP and GTP (not shown) as phosphate donors. As expected, the mitochondrial fraction of parental cell lines contained the 0.7-0.8 Rm dT kinase, and, in addition, a second minor dT kinase activity with an Rm of about 0.4 (Fig. 2b, 3b). The 0.4 Rm dT kinase activity utilized only ATP as a phosphate donor.

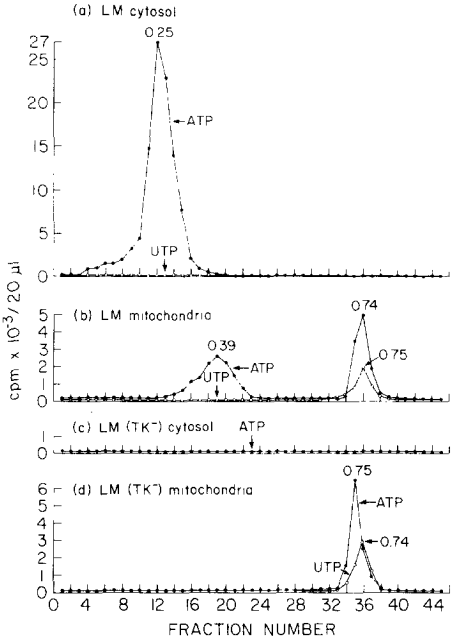

Figure 3: Disc PAGE analyses of dT kinase activities of: (a) LM cytosol; (b) LM mitochondrial; (c) LM(TK$^-$) cytosol; and (d) LM(TK$^-$) mitochondrial fractions. See legend of Fig. 2 for experimental details.

Although the 0.4 Rm mitochondrial dT kinase activity of parental cells might be a separate isozyme, we tentatively favor the hypothesis that the 0.4 Rm activity is a modified form of the cytosol dT kinase. Results analogous to those with mouse lines have been obtained with dT kinase-deficient, human HeLa(BU25) and parental HeLa S3 cells (7). The HeLa(BU25) mitochondrial dT kinase differs from the cytosol enzyme of HeLa S3 and other human cell lines in disc PAGE patterns, phosphate donor specificity, sedimentation coef-

ficient, pH-activity curves, and in sensitivity to inhibition by dCTP (5,7).

Our experiments suggest that a distinctive dT kinase isozyme exists in mouse mitochondria. The characteristic properties of the mitochondrial dT kinase may facilitate studies on the site of synthesis and mechanism of control of this enzyme. This dT kinase marker may also be useful in studies of mitochondrial biogenesis.

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