EFFECT OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON THE FORMATION OF MITOCHONDRIAL-SPECIFIC THYMIDINE KINASE ISOZYMES IN HeLa(BU25) CELLS

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Received September 2,1975

SUMMARY: HeLa(BU25) cells, though deficient in cytosol thymidine kinase-F activity, contain 2 mitochondrial thymidine kinase molecular forms, designated thymidine kinase A and thymidine kinase B. The formation of thymidine kinase A activity is cycloheximide-sensitive and chloramphenicol-resistant, while the reverse applies to the formation of thymidine kinase B. Formation of cytosol thymidine kinase F in wild-type and chloramphenicol-resistant HeLa S3 cells is also cycloheximide-sensitive and chloramphenicol-resistant.

INTRODUCTION: Bromodeoxyuridine-resistant HeLa(BU25) cells contain significant amounts of 2 mitochondrial TK[1] isozymes, designated TK-A and TK-B, despite the loss of the principal cytosol isozyme (TK-F) from the mutant cells (1,2). The mitochondrial TK-A and TK-B molecular forms differ in Rm, isoelectric point, sedimentation coefficient, phosphate donor specificity, and sensitivity to dCTP inhibition, and they can also be distinguished from the cytosol TK-F isozyme of wild-type HeLa S3 cells. Furthermore, TK-A is a deoxypyrimidine kinase - that is, it catalyzes the phosphorylation of both dT and dC, while mitochondrial TK-B and cytosol TK-F catalyze the phosphorylation of dT but not dC (3). TK-A activity is localized in the mitochondrial matrix fraction. In contrast, TK-B activity, though detectable in the mitochondrial matrix, is found primarily in the mitochondrial inner membrane fraction (4).

Studies on somatic cell hybrids have shown that the determinants for mitochondrial TK-A and cytosol TK-F are on different linkage groups (5). However, the origin of TK-B and its relationship to the other TK isozymes is obscure. To gain insight into the origin of TK-B activity, the time-course of

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[1] Abbreviations used: TK, thymidine kinase; CH, cycloheximide; CAP, chloramphenicol; Rm, electrophoretic mobility relative to the tracking dye.

formation of this molecular form was investigated in cell cultures treated with CAP and CH. Studies in yeast, Neurospora, Tetrahymena, and mammalian cells have shown that CAP and CH inhibit mitochondrial- and cytosol-specific protein synthesis, respectively (6-11). The data to be presented demonstrate that formation of TK-B activity is inhibited by CAP, but not CH, while the reverse is true of the appearance of TK-A activity.

MATERIALS AND METHODS: Wild-type HeLa S3 and mutant HeLa(BU25) cells were grown in Eagle's minimal essential medium supplemented with 10% calf serum (1,2,12). For studies on the effects of drugs on cell growth and TK isozyme formation, replicate cultures were seeded at 10^6 cells per 8 oz prescription bottle and grown for 2 days without drugs. The medium of control and experimental cultures was changed and CAP or CH was added to experimental cultures at final concentrations of 50 and 10 μg/ml, respectively. The medium was again changed 4 days after seeding. Two days after seeding and at various times after drug treatment, cultures were harvested by trypsinization, and mitochondrial or cytosol extracts prepared (2,3). TK activity was assayed using ATP as phosphate donor and ^{3}H-dT as nucleoside acceptor (3). The disc PAGE analysis of TK molecular forms has been described (1).

RESULTS AND DISCUSSION: Although the vast majority of mitochondrial proteins are synthesized on cytoplasmic ribosomes and transported into the mitochondria in a subsequent step, the proteins synthesized within the mitochondria are essential for mitochondrial inner membrane formation (7). CAP is one of a number of antibiotics which specifically inhibits mitochondrial protein synthesis in eukaryotic cells, while not significantly affecting the cytoplasmic system (6-11). CAP inhibits overall protein synthesis by HeLa S3 mitochondria *in vivo* and *in vitro* (13,14). In yeast, CAP-resistant mutants manifest alterations in mitochondrial membrane permeability and in mitochondrial ribosomal proteins (10). In CAP-resistant HeLa(296-1) mitochondria, however, the CAP mutation(s) is unlikely to be one affecting only permeability. The mutation(s) affects the mitochondrial protein synthesizing system itself and/or an enzyme

TABLE I

Effects of Chloramphenicol (CAP) and Cycloheximide (CH) on the Growth and Mitochondrial Thymidine Kinase (TK) Activities of HeLa(BU25) and Cytosol TK Activity of HeLa S3 Cells

Enzyme source	Days after seeding	Cells (10^6) per culture: Control	CAP	CH	Total TK activity* per culture: Control	CAP	CH	TK activity* per 10^6 cells: Control	CAP	CH	TK activity* per µg protein: Control	CAP	CH
HeLa(BU25) Mitochondria	2	4.4	-	-	103	-	-	23.2	-	-	0.9	-	-
	3	6.8	5.6	-	167	153	-	24.4	27.4	-	1.5	1.3	-
	4	11.3	8.8	-	285	202	-	25.2	22.9	-	1.5	1.3	-
	5	18.4	10.7	-	498	265	-	26.9	24.8	-	1.9	1.9	-
	6	24.7	14.8	-	635	374	-	25.7	25.2	-	1.7	2.3	-
HeLa(BU25) Mitochondria	2	2.1	-	-	94	-	-	44.5	-	-	2.2	-	-
	3	6.6	-	1.9	252	-	177	38.1	-	93.3	1.9	-	4.6
	4	10.7	-	1.6	416	-	205	38.9	-	132.0	1.2	-	5.1
HeLa S3 cytosol	2	3.0	-	-	2710	-	-	904	-	-	8.8	-	-
	3	6.7	-	2.1	9430	-	1690	1400	-	797	12.1	-	8.8
	4	11.6	-	1.8	9320	-	47	804	-	234	7.6	-	4.7

* Picomoles dTMP formed in 60 min at 38°.

activity which inactivates CAP (11,13).

To learn whether mitochondrial or cytoplasmic protein synthesizing systems were needed for the formation of the HeLa(BU25) mitochondrial TK molecular forms, experiments in which cells were cultivated in the presence of CAP or CH were carried out (Table I). The following points may be noted: (1) CH-treatment completely prevented HeLa(BU25) cell growth, but CAP only gradually inhibited cell growth, so that after 4 days of CAP treatment, the HeLa(BU25) cell number per culture was about 60% of that of untreated cultures; (2) the total mitochondrial TK activity increased about 6-fold from 2-6 days after seeding in control cultures and about 4-fold in CAP-treated cultures; (3) total mitochondrial TK activity also increased despite 48 hr of CH treatment; (4) the TK activity per 10^6 cells was relatively constant in control and CAP-treated cultures, but increased in cultures treated for 2 days

with CH; (5) the TK activity per μg protein was also similar in control and CAP-treated cultures, but more than doubled in CH-treated cultures.

Disc PAGE analyses of HeLa(BU25) mitochondrial extracts from control and drug-treated cells are shown in Fig. 1. The TK-A and TK-B molecular forms exhibit Rm values of about 0.6 and 0.4, respectively. Figure 1 illustrates that the ratios of the two activities changed markedly after cultures were treated with CAP or CH.

The total enzyme activities per culture of each of the mitochondrial TK molecular forms are plotted in Fig. 2 as a function of time after drug treatment. Both the TK-A and TK-B molecular forms increased in parallel with the increase in cell number in control cultures. In CAP-treated cultures, TK-B activity decreased 1 day after drug treatment, but TK-A activity in-

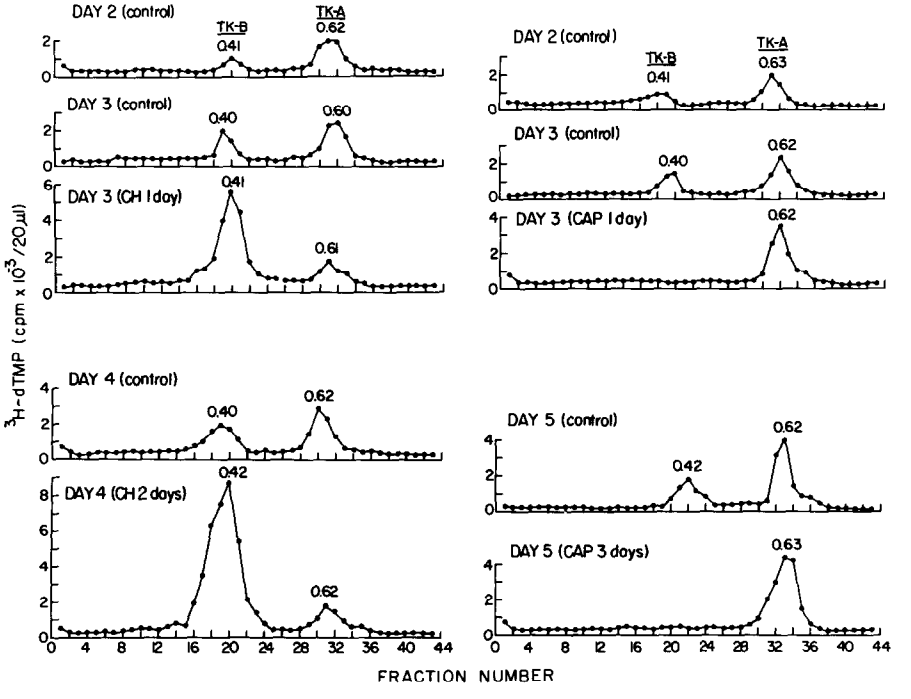

Figure 1: Disc PAGE analyses in 5% polyacrylamide gels of mitochondrial extracts from untreated and from CAP- or CH-treated HeLa(BU25) cells. Two days after seeding, CAP (50 μg/ml) or CH (10 μg/ml) was added to experimental cultures. The cultures were harvested at the times after seeding shown in the figure. Numbers above the peaks signify Rm values.

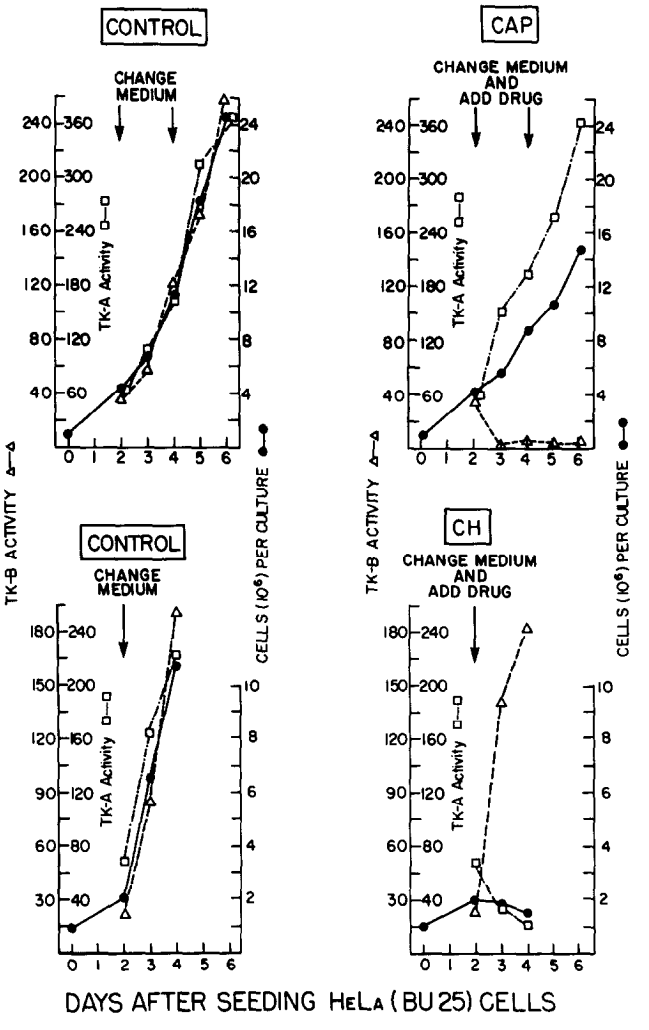

Figure 2: Effects of CAP- and CH-treatment on the formation of mitochondrial TK molecular forms of HeLa(BU25) cells. Enzyme extracts were analyzed by disc PAGE. The TK-A and TK-B activities per culture were calculated from the data of Table I and Fig. 1 and plotted as indicated above. Activity expressed as picomoles dTMP formed in 60 min at 38° per culture.

creased faster than the number of cells per culture. In contrast, in CH-treated cultures, TK-B activity increased substantially despite the inhibition of cell growth, but TK-A activity decreased.

The formation of cytosol and mitochondrial TK isozymes in CAP-resistant HeLa(296-1) cells was also studied (11,13). Whether propagated in the presence or absence of CAP: (1) the CAP-resistant HeLa(296-1) cells did not exhibit a

mitochondrial TK-B activity, but they did contain approximately the same levels of cytosol TK-F and mitochondrial TK-A as found in wild-type HeLa S3 cells; and (2) their cytosol TK-F and mitochondrial TK-A activities increased in parallel with the increase in cell number (data not shown).

Experiments on the formation of cytosol TK-F in cultures of CH-treated wild-type HeLa S3 cells are shown in Table I. In control cultures, the total cytosol TK-F activity per culture increased about 4-fold at 2-4 days after subculture. In CH-treated cultures, total cytosol TK-F activity per culture decreased about 40% and 98% after 1 and 2 days of drug treatment, respectively.

The experiments described in this study strongly support the hypothesis that mitochondrial TK-A is coded by nuclear genes, translated on cytoribosomes, and subsequently translocated to the mitochondrial matrix. Cytosol TK-F is coded by human chromosome E17, synthesized on cytoribosomes, and small amounts of TK-F are also translocated to the mitochondrial matrix of HeLa S3 and HeLa(296-1) cells. However, mitochondrial TK-B requires mitochondrial protein synthesis for activity. The experiments suggest that: (1) mitochondrial DNA codes for TK-B; or (2) the cells harbor an unknown virus or bacterium which codes for TK-B. With regard to these possibilities, the following points are pertinent: (1) if a subunit of TK-B were translated on cytoribosomes, CH would be expected to inhibit the appearance of TK-B activity, and this is not observed; (2) the HeLa(BU25) and HeLa S3 cell lines have been tested periodically for mycoplasma contamination, but none has been detected; and (3) none of the 3 TK isozymes exhibit significant nucleoside phosphotransferase activity, as might be expected if one of the isozymes were determined by an endogenous mycoplasma or phosphotransferase-positive bacteria in the cultures.

The experiments on drug-treated HeLa(BU25) cells suggest convenient procedures for the preparation of the TK-A isozyme with minimal contamination by the TK-B activity and vice versa. These procedures should facilitate the purification of the isozymes and the study of the incorporation of radioactive amino acids into their respective polypeptides.

ACKNOWLEDGMENTS: This investigation was aided by Robert A. Welch Foundation Grant Q-163 and by USPHS Grants CA-06656-13, CA-10893 and 1-K6-AI-2352 from the National Cancer Institute and the National Institute of Allergy and Infectious Diseases. We thank Mary D'Anton, Maureen Kaough, Geraldine Jorgensen and Sarah Coombs for technical assistance.

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