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Cell cycle regulation of deoxycytidine kinase

Evidence for post-transcriptional control

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Deoxycytidine kinase enzyme activity and deoxycitidine kinase mRNA content were determined at different positions of the cell cycle in permanent cell lines. There was no variation of deoxycytidine kinase activity during the cell cycle in two cell lines, whereas in two other lines the enzyme activity was induced (10- and 15-fold) at the G1/S boundary. In contrast, the level of deoxycytidine kinase mRNA never displayed any cell cycle-dependent changes. The decay of enzymatic activity was measured after addition of cycloheximide in different phases of the cell cycle; the enzyme was much more stable in cells with constant activity. We suggest that post-transcriptional mechanisms account for the periodic behaviour of the enzyme activity, and that whether this regulation can be detected depends on the half-life of deoxycytidine kinase.

Deoxycytidine kinase; Cell cycle regulation; Post-transcriptional control

1. INTRODUCTION

Deoxycytidine kinase (dCK; EC 2.7.1.74) catalyzes the phosphorylation of deoxycytidine to deoxycytidine 5′-monophosphate using triphosphates as phosphate donors. This enzyme has a broad substrate specificity [1,2] and plays a physiologic role in the maintenance of normal deoxyribonucleotide pools. It is responsible for the initial activation of a number of clinically important chemotherapeutic and antiviral agents, such as 1-β-D-arabinofuranosylcytosine (Ara-C), 2-fluoro-9-β-D-arabinofuranosyladenine, and 2′,3′-dideoxycytidine [3,4]; deficiency of dCK activity causes resistance to these drugs.

The cDNA for this enzyme has been cloned and the corresponding amino acid sequence of the protein subsequently predicted [5]. Very recently the structure and chromosomal localization of the human dCK gene has been published [6], which is the basis for obtaining further insight into the regulation of its expression.

In eukaryotic cells deoxyribonucleoside triphosphate biosynthesis occurs periodically in close relation with DNA replication. Enzymes responsible for the biosynthesis of deoxyribonucleotides, such as ribonucleotide reductase, thymidylate synthetase, deoxycytidine deaminase, uridine kinase, dihydrofolate reductase, and thymidine kinase, are essentially synthesized during S phase of the cell cycle (reviewed in [7]). Although the salvage pathway enzyme dCK also belongs to this group of enzymes, its activity was believed to be much less influenced by progression through the cell cycle phase. Earlier studies of Pregoraro and Bernengo [8] and Arner et al. [9] demonstrated a high dCK activity (~ 30 pmol/mg/min) in resting lymphocytes and a modest (2- to 3-fold) increase of activity after phytohemagglutinin stimulation. Very similar observations were made after serum stimulation of BHK21/C13 cells; whereas thymidine kinase activity increased 20-fold from a very low initial level after stimulation, dCK activity remained at a constant high level [10]. In addition, Richel et al. [11] could not detect any variation of dCK activity during the cell cycle of elutriated HL-60 cells.

Recently it has been demonstrated [5,6] that the steady state level of dCK mRNA varies in different cell types. For more detailed characterization of the regulation of dCK expression, we studied enzyme activity and mRNA levels during the cell cycle of various permanent cell lines. This work presents evidence for post-transcriptional regulation of deoxycytidine kinase expression during the eucaryotic cell cycle.

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2. MATERIALS AND METHODS

2.1. *Cells*

3T6 cells (permanent line of contact inhibited mouse fibroblasts; ATCC CCL96) and COP-8 cells (polyoma transformed mouse cells) were grown on plastic Petri dishes in Dulbecco's modified Eagles's (DME) medium supplemented with 10% calf serum. HeLa cells (human cervix carcinoma; papilloma transformed cels; ATTC CCL2) and Epstein Barr virus-transformed human lymphocytes (EBV lymph) were cultivated in 75-cm^2 flasks containing RPMI medium with 10% calf serum. All tissue cultures were maintained at 37°C and 5% CO_2, and were routinely screened for the absence of mycoplasma.

Cells were fractionated with a JE-6B Elutriator in a J2-21M centri-

fuge (Beckman) as previously described [12]. Medium flow was controlled with a Cole-Parmer Masterflex pump and the rotor was kept at a speed of 2,000 rpm. The elutriation medium was phosphate-buffered saline (PBS) supplemented with 0.9 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$ and 2% calf serum. Each elutriated fraction was monitored by a PAS-II flow cytometer (Partec) using 6 μmol/l 4,6-diamidino-2-phenylindol-dihydrochlorid (DAPI) (Merck) to stain DNA.

2.2. *Deoxycytidine kinase assay*

Samples were diluted in the sample buffer as described [13] and the protein concentration in these extracts were measured by the method of Bradford [14] using the Bio-Rad protein assay reagent with bovine serum albumin as a standard. Enzyme activity was determined by a method described by Ives and Wang [13]. dCK activity was measured with [5-^{3}H]deoxycytidine (0.5 μCi; spec. act. 25.6 Ci/mmol) as substrate in a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM ATP, 2 mM DTT, 10 mM NaF and about 0.5 mg/ml of cell extract. Samples were incubated at 37°C, 15 μl were spotted on DEAE cellulose filters which were washed and counted.

2.3. *RNA extractions and Northern blotting*

RNA was isolated after cell lysis in an isotonic buffer containing the nonionic detergent NP40 and after removal of nuclei by centrifugation [15]. 20 μg of each sample was electrophoresed in a 1% formaldehyde agarose gel. RNA was transferred to a nylon membrane, hybridized sequentially with ^{32}P-radiolabeled probes specific for dCK and β2-microglobulin sequences, and the ratio determined by densitometry.

2.4. *dCK cDNA probe*

We synthesized a 23mer sense oligonucleotide (position 734–756 bp of the dCK cDNA) and a 22mer antisense oligonucleotide (position 1422–1443 bp), both homologous to the dCK cDNA published by Chottiner et al. [5]. Using these oligonucleotides as primers for the polymerase chain reaction, templated by cytoplasmic RNA of the cell line MOLT-4 (ATTC CRL1582), we amplified a 710 bp cDNA fragment, which could be identified as dCK-specific by two *Hin*dDIII cleavage sites. Hybridization with this probe revealed a single 2.8 kb mRNA as described for dCK [5].

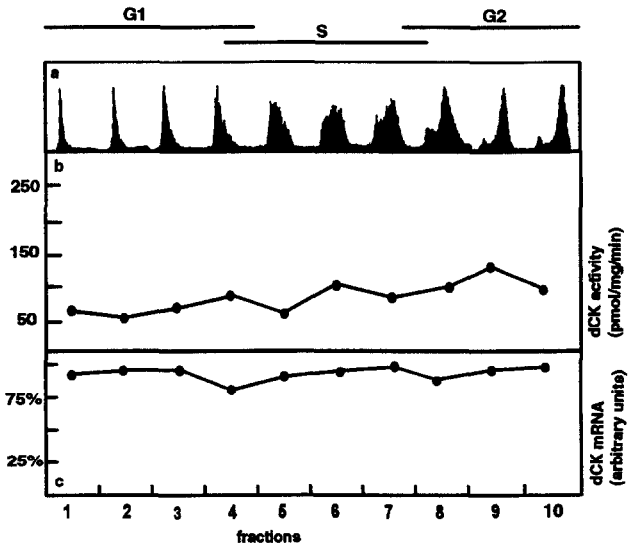

Fig. 1. dCK activity vs. mRNA content during the cell cycle of elutriated 3T6 mouse fibroblasts. Cells were separated into fractions of different cell cycle position by centrifugal elutriation as indicated at the top (G1, S and G2). (a) DNA content was quantitated by flow cytometry after staining with DAPI. (b) dCK activity is given in pmol dCMP formed/mg protein/hour. (c) dCK mRNA is normalized to β2-microglobulin and expressed in relative units (the highest value = 100%.

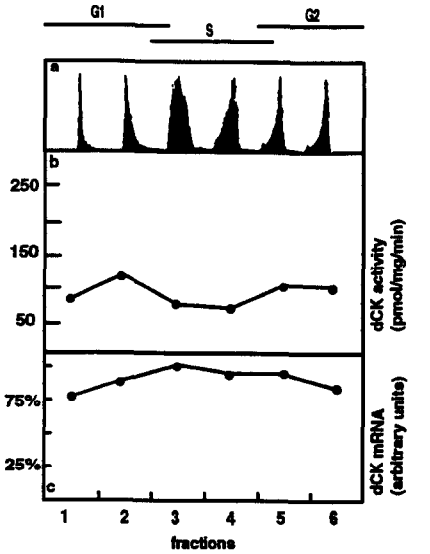

Fig. 2. dCK activity vs. mRNA content during the cell cycle of elutriated Epstein Barr virus-transformed lymphocytes. (a) DNA content. (b) dCK enzyme activity. (c) dCK mRNA level. See Fig. 1 for more detailed information.

3. RESULTS

3.1. *dCK enzyme activity during the cell cycle*

Four permanent lines were separated according to their phase of the cell cycle by centrifugal elutriation. During the elutriations of HeLa cells and EBV-transformed lymphocytes the medium flow rates were changed in larger steps. The cell cycle of these cells were divided in only six fractions (compared to nine and ten in COP-8 and 3T6) to be sure to get enough cells per fraction for the analyses of dCK activity and mRNA level.

As the DNA distribution of the collected fractions indicate, the separation quality of all four elutriations shown in Figs. 1–4 was comparable. The first fraction reflects a distribution of about 88% G1-, 10% S- and 2% G2/M-cells. The best S-phase fractions derived from each of the four cell lines also had comparable amounts of cells of different cell cycle position (17% G1, 70% S, 13% G2/M). Only the G2/M fractions of COP-8 cells contained a higher amount of G1 and S phase cells than the other cell lines. The average distribution of the best G2/M fractions of 3T6 mouse fibroblasts, HeLa cells and EBV transformed lymphocytes was 8% G1, 15% S and 77% G2/M.

3T6 mouse fibroblasts and human EBV-transformed lymphocytes do not display any cell cycle regulation of dCK enzyme activity (see Fig. 1b and Fig. 2b). In both cell lines the activity only varies between 50 and 100 pmol dCMP formed/mg protein/min during the cell cycle. This is in agreement with the measured dCK activities in logarithmically growing 3T6 cells and virus transformed lymphocytes (see Table I).

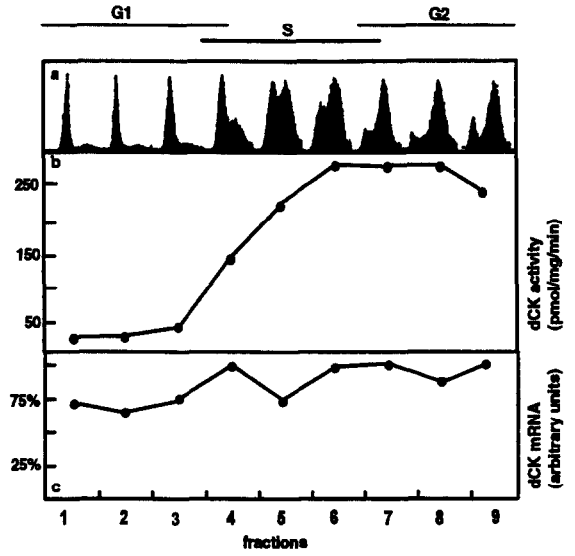

Fig. 3. dCK activity vs. mRNA content during the cell cycle of the permanent cell line COP-8 (polyoma-transformed mouse fibroblasts). (a) DNA content. (b) dCK enzyme activity. (c) dCK mRNA level. See Fig. 1 for more detailed information.

In G1 cells of HeLa and COP-8 the dCK activity is very low. A 10- and 15-fold induction of dCK activity appeared at the G1/S boundary of the cell cycle of these cells. During the G2 phase of these two lines the activity slightly decreased, but mainly kept the elevated level. This G1/S induction to a high dCK activity in S and G2 could be an explanation for the high enzyme activity in logarithmically growing cells (compare Table I).

3.2. *dCK mRNA level during the cell cycle*

We also determined the dCK mRNA level in each separated fraction. The obtained values were normalized to β2-microglobulin mRNA, which is well known to be constitutively expressed. As seen in Figs. 1–4c, no cell cycle regulation of dCK mRNA could be detected. The mRNA level of this enzyme increased during the cell cycle of COP-8 cells. As this induction is less than 1.5-fold, it does not indicate a cell cycle regulation.

3.3. *dCK enzyme stability*

After centrifugal elutriation separated G1, S and G2/M cells were recultivated in the presence of cycloheximide. 0, 1.5, 3, 4.5 and 6 h after recultivation dCK activity was measured and the half-life of enzyme activity in the different cell cycle phases was determined (Table I). In no studied lines was cell cycle-dependent variation of the dCK stability detected. In 3T6 mouse fibroblasts and in EBV-transformed lymphocytes, the enzyme is constitutively more stable (3- to 4-fold) than in COP-8 and HeLa cells. Interestingly, the average of enzyme activity in logarithmically growing cells was 2-fold higher in HeLa cells and COP-8 cells than in 3T6 cells and transformed lymphocytes.

4. DISCUSSION

As deoxyribonucleoside triphosphate biosynthesis is closely connected with DNA replication, the enzymes responsible for this replication are mainly expressed during the S phase of the eucaryotic cell cycle [7]. The salvage pathway enzyme deoxycytidine kinase also belongs to DNA precursor pathway enzymes, but its activity was shown to be cell cycle independent [8–11].

We suggest that it depends on the cell type, whether dCK activity increases during S phase or not. In the two permanent cell lines COP-8 (polyoma-transformed mouse cells) and HeLa (human cervix carcinoma; papilloma-transformed) a 10- and 15-fold induction at the G1/S boundary was detected. In contrast 3T6 mouse fibroblasts and human Epstein Barr virus (EBV) transformed lymphocytes do not display any cell cycle regulation of dCK activity. Interestingly, cell cycle-dependent variations of the steady-state mRNA level could not be detected. This fact suggests a post-transcriptional control of dCK expression during the cell cycle. When measured in the presence of cycloheximide, the enzyme is much more stable in the non-regulated cells compared to the regulated cases. It seems that the post-transcriptional regulation of dCK observed in COP-8 and HeLa cells cannot be determined in 3T6 cells or EBV transformed lymphocytes, because of the longer half-life of the enzyme. Once an antibody for dCK is available it would be interesting to determine the half-life of the protein during the eucaryotic cell cycle.

Sherley and Kelly [16] defined the molecular basis for the periodic expression of thymidine kinase activity during the eucaryotic cell cycle. Studying HeLa cells they showed that the large induction of thymidine kinase activity on the G1/S boundary is not paralleled at the

Table I

Half-life of dCK enzyme activity in different cell cycle phases in the presence of cycloheximide and dCK activities in logarithmically growing cells

Cell line	Half-life of dCK enzyme activity in hours			dCK activity in logarithmically growing cells*
	G1	S	G2/M	
EBV lymph	13.1 ± 1.2	12.8 ± 0.8	11.6 ± 1.7	82.3 ± 11.8
3T6	11.2 ± 1.4	9.8 ± 2.3	10.9 ± 2.2	74.5 ± 23.5
COP-8	2.5 ± 0.9	3.2 ± 1.4	2.8 ± 1.4	153.4 ± 33.9
HeLa	2.2 ± 1.1	2.8 ± 2.0	3.5 ± 0.5	149.6 ± 19.1

Cells were separated by centrifugal elutriation according to their size. Fractions representative for G1, S, or G2 phase of the cell cycle were re-cultivated in the presence of 10 μg/ml cycloheximide, At 0, 1.5, 3, 4.5, and 6 h after re-cultivation, dCK activities were measured. Half-lifes of dCK activity in the different cell cycle phases were determined. Values are means of three independent measurements ± S.D.

*The dCK activities in logarithmically growing cells are given in pmol dCMP formed/mg protein/min. (EBV lymph, Epstein Barr virus-transformed human lymphocytes).

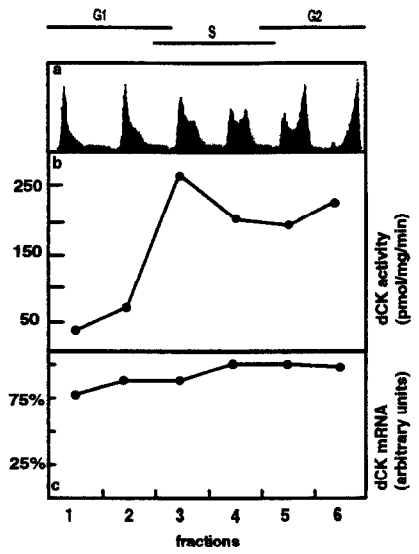

Fig. 4. dCK activity vs. dCK mRNA content during the cell cycle of elutriated HeLa cells. (a) DNA content. (b) dCK enzyme activity. (c) dCK mRNA level. See Fig. 1 for more detailed information.

mRNA level. The authors postulated a 10-fold greater efficiency of translation of thymidine kinase mRNA when cells begin DNA replication. In addition, the stability of thymidine kinase protein dramatically decreased upon cell division.

Studying deoxycytidine kinase activity and mRNA in HeLa cells and in polyoma-transformed mouse cells (COP-8) revealed a comparable regulation. Whereas the steady-state mRNA level is not cell cycle-regulated, the dCK enzyme activity is induced 10- and 15-fold at the G1/S boundary. In this work we show that the dCK enzyme stability does not vary between G1-, S- and G2/M-phase of these two cell lines. However, we do not know if there exists an increased protease activity degrading deoxycytidine kinase during metaphase of mitosis, as is postulated for thymidine kinase [17]. One may speculate that a dramatically decreased stability during mitosis, as described for thymidine kinase, could lead to the observed very low dCK activity in G1 phase of HeLa and COP-8 cells.

Our data indicate that this difference in dCK enzyme stability seems not to be a marker for transformation, as EBV-transformed lymphocytes and HeLa cells (cervix carcinoma; papilloma-transformed) display a totally different dCK stability. It would be interesting to look for a correlation between a specific kind of transformation and the observed changes of dCK regulation. Certainly we showed that the variation in dCK stability is not a difference between mice and man.

Several studies have demonstrated that dCK is expressed at variable levels in different tissues. The activity of this enzyme in leukemic T lymphoblasts and in thymus glands has been shown to be higher than in other cell types (reviewed in [6]). Maybe the two observed dCK enzyme stabilities play an additional role in these different levels of expression.

Because of the potential role for this enzyme in mediating the tissue-specific cytotoxicity of chemotherapeutic agents, further characterization of the factors regulating dCK expression should be done. An important question to answer (e.g.) would be: is the observed difference in enzyme stability specific for dCK or are other DNA precursor pathway enzymes similarly affected?

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