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Mitochondrial Deoxyribonucleoside Salvage Enzymes

Cloning and characterization of deoxyguanosine kinase and thymidine kinase

Liya Wang

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Abstract

Mitochondrial deoxyguanosine kinase (dGK) and thymidine kinase (TK2) are responsible for the phosphorylation of deoxyribonucleosides, thus providing DNA precursors for mitochondrial DNA synthesis. Bovine dGK was purified 20,000-fold to apparent homo-geneity. The purified dGK was capable of phosphorylating all the natural occurring purine deoxyribonucleosides, and a number of clinically important anticancer nucleoside analogs, *e. g.* CdA (2-chloro-2'-deoxy-adenosine) and araG (9-β-D-arabinofuranosylguanine). A selective assay method for dGK determination was developed by using araG as substrate.

dGK activity levels were elevated in certain brain tumors which may have implications for future anticancer chemotherapy.

Amino aicd sequence information from purified bovine dGK was used in RT-PCR to amplify a dGK cDNA fragment. The entire dGK cDNA was subsequently cloned from a human brain cDNA library, and it encoded a 30 kDa protein. The peptide sequences from purified bovine dGK were identified in the deduced amino acid sequence and when expressed in *E. coli.* the recombinant protein catalysed efficient phosphorylation of dGuo, araG, CdA and dIno similar to purified bovine dGK. Northern blot analysis demonstrated one dominant transcript of 1.35 kb which was found in all tissues at similar levels.

A similar approach was used to clone the cDNA for TK2. A TK2 cDNA was cloned from a human brain cDNA library and RACE PCR was used to amplify the cDNA ends from human brain mRNA. All the peptide sequences were found in the deduced amino aicd sequence. Expression of N-terminal truncated TK2 in *E. coli* yieled a fully active TK2 protein of 25.5 kDa which showed enzymatic properties identical to the purified human and bovine proteins. Northern blot analysis using coding sequence of TK2 cDNA demonstrated several mRNAs, the major ones were 2.2 and 4.0 kb and they were found in most tissues at similar levels.

Both dGK and TK2 cDNA sequences show great similarities to the cytosolic deoxycytidine kinase sequence, about 40% at the amino acid level, suggesting that they are evolutionary related and thus established a new enzyme family of mammalian deoxynucleoside kinases.

Keywords: mitochondrial DNA precursor synthesis, deoxyguanosine kinase, mitochondrial thymidine kinase, deoxycytidine kinase, herpes virus thymidine kinase, chemotherapy, nucleoside analogs, araG (9-β-D-arabinofuranosylguanine), CdA (2-chloro-2'-deoxyadenosine), cDNA, sequence motifs.

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Doctoral thesis
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To my mother

Abstract

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Contents

Papers on which this thesis is based

Papers I–IV

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Wang, L., Karlsson, A., Arnér, E. S. J. & Eriksson, S. 1993. Substrate specificity of mitochondrial 2'-deoxyguanosine kinase: Efficient phosphorylation of 2-chlorodeoxyadenosine. *J. Biol. Chem. 268,* 22847-22852.

II. Wang, L., Karlsson, A., Mathiesen, T. & Eriksson, S. 1993. 2-Chloro-2'-deoxyadenosine phosphorylation by deoxyguanosine kinase in crude extracts of malignant human brain tissue. *Recent Advances in Chemotherapy* (ed. Einhorn, J., Nord, C. E., & Norrby, S. R.). Proceedings of the 18th International Congress of Chemotherapy, Stockholm, Sweden, American Society for Microbiology, 919-921.

III. Wang, L., Hellman, U. & Eriksson, S. 1996. Cloning and expression of human mitochondrial deoxyguanosine kinase cDNA. *FEBS lett. 390,* 39-43.

IV Wang, L., Herrström Sjöberg, A., Bergman, T., Hellman, U. & Eriksson, S. 1996. Cloning and expression of human mitochondrial thymidine kinase cDNA. *(Manuscript)*

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Abbreviations

The following abbreviations are used in the text.

Enzymes:

dGK	Mitochondrial deoxyguanosine kinase
TK2	Mitochondrial thymidine kinase
dCK	Cytosolic deoxycytidine kinase
TK1	Cytosolic thymidine kinase
HSV TK	Herpes virus thymidine kinase
AK	Adenosine kinase
5'-NT	5'-nucleotidase
PNP	Purine nucleoside phosphorylase
ADA	Adenosine deaminase

(Deoxy)ribonucleosides and (deoxy)ribonucleotides and analogs

dAdo, dAMP, dADP, and dATP	Deoxyadenosine, mono-, di-, and triphosphate
dGuo, dGMP, dGDP, and dGTP	Deoxyguanosine, mono-, di-, and triphosphate
dCyd, dCMP, dCDP, and dCTP	Deoxycytidine, mono-, di-, and triphosphate
Thd, dTMP, dTDP, and dTTP	Deoxythymidine, mono-, di-, and triphosphate
dUrd, dUMP, dUDP, and dUTP	Deoxyuridine, mono-, di-, and triphosphate
dIno, dIMP	Deoxyinosine, monophosphate

Ado, AMP, ADP, ATP	Adenosine, mono-, di-, and triphosphate
CTP	Cytosine triphosphate
UTP	Uridine triphophate
GTP	Guanosine triphosphate

ddI	2',3'-dideoxyinosine
ddA	2',3'-dideoxyadenosine
AZT	3'-azido-2'-deoxythymidine
FdUrd	5-fluoro-2'deoxyuridine
CdA	2-chloro-2'-deoxyadenosine
araG	9-β-D-arabinofuranosyl guanine
araA	9-β-D-arabinofuranosyl adenine
araC	9-β-D-arabinofuranosyl cytosine
araT	9-β-D-arabinofuranosyl thymine
araU	9-β-D-arabinofuranosyl uracil
araHx	9-β-D-arabinofuranosyl hypoxanthine
BvdU	5-(2-bromovinyl)-2'-deoxyuridine
ACV	acyclovir, 9-(2-hydroxyethoxymethyl)guanine
GCV	gancilovir, 9-(1,3-dihydroxy-2-propoxymehty)guanine
FIAU	1-(2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil

ORF	open reading frame
bp	base pairs
kb	kilo base pairs

General introduction

This thesis is mainly focused on the enzymes involved in the DNA precursors metabolism, particularly the mitochondrial deoxyribonucleoside salvage enzymes, *i.e.* mitochondrial thymidine kinase and mitochondrial deoxyguanosine kinase. These two enzymes are at least in part responsible for the supply of deoxyribonucleotides for mitochondrial DNA synthesis, especially in non-proliferating cells which lack the *de novo* synthesis of deoxynucleotides. They are also involved in activation of clinically used nucleoside analogues, *e.g.* arabinosylguanine and 2-chloro-2'-deoxyadenosine, and they contribute to their therapeutic effects as well as their toxic side effects. Deoxyguanosine kinase may also play an important role in severe combined immunodeficiency disease in man caused by purine nucleoside phosphorylase deficiency. As a general background to this study a summary of the properties and regulation of enzymes involved in the DNA precursor metabolism will be presented.

DNA replication – mitochondrial DNA synthesis

In mammalian cells, DNA replicates at two locations: the nucleus and the mitochondrion. Nuclear DNA replicates only during S phase before cells enter mitosis. The event of nuclear DNA synthesis only occurs in proliferating cells and not in resting or terminally differentiated cells. This is probably achieved by regulation of the expression of key enzymes. For instance, DNA polymerase α, a key DNA replicating enzyme, is located in the nucleus and its expression is strictly cell cycle controled (Weissbach, 1977; Chang and Bollum, 1972; Chiu and Baril, 1975). The synthesis of mitochondrial DNA (mtDNA), on the other hand, is not co-ordinated to nuclear DNA synthesis. Mitochondria is the energy-converting organelle in the cell. In man, mitochondria contain multiple, closed-circular DNA molecules of 16569 bp. The ratio of mitochondrial to cellular volume is constant, as is the number of mitochondrial genoms (Shay *et al.*, 1990). Generally there is about 2% of total cellular DNA as mtDNA, while in the oocytes 50% of all DNA is mtDNA. In most cells, mitochondria divides throughout interphase, out of phase with either the division of the cells or with each other to meet the needs of cell division and rapid mitochondrial turnover. Similarly, the replication of mtDNA is not limited to S-phase, but occurs throughout the cell cycle (Heintz and Stillman, 1988). Correlated with the expression of the enzyme responsible for mtDNA synthesis, DNA polymerase gamma (pol γ), which is encoded by a nuclear gene, is constitutively expressed (Shioda, 1988). It is known that disturbances of DNA precursor pool synthesis can be mutagenic. In man, mutations in nuclear DNA (of functional genes) may lead to severe defects and even to cell death, while mutations in mt DNA are most likely involved in a wide spectrum of degenerative disorders (Wallace, 1992). The maintenance

of sufficient DNA precursor pools is of importance to ensure the replication, repair and expression of both the nuclear and the mitochondrial genome.

The synthesis of DNA precursors

The de novo pathway

In the *de novo* synthesis pathway, cells utilise small molecules such as CO_2, amino acids and vitamins to synthesize ribonucleotides (ribonucleoside mono-, di- and triphosphate). Deoxyribonucleotides are formed by reduction of the 2'-hydroxyl group of the corresponding ribonucleoside diphosphate in a reaction catalyzed by ribonucleotide reductase (EC 1.17.4.1). The *de novo* pathway is under strict control by the allosteric regulation of ribonucleotide reductase and by its cell cycle dependent expression (for review see Thelander and Reichard, 1979; Reichard, 1988; Stubbe, 1990; Elledge *et al.*, 1992; Greenberg and Hilfinger, 1996). The active ribonucleotide reductase is composed of two dissimilar subunits, R1, the large subunit containing two allosteric sites, and R2, the small subunit containing the binuclear iron center and a tryosyl free radical. The allosteric control is manifested by the competitive binding of the end products, the dNTP's, to the substrate specificity site or the activity site on R1. The expression of this enzyme is also tightly regulated. Interestingly, R1 and R2 proteins have different half lives. R1 has a half life of about 20 hr and it is present throughout the cell cycle in proliferating cells (Engström *et al.*, 1985) and the S-phase specific regulation is due to the short half life (about 3 hr) of the R2 protein which is expressed only during S phase (Eriksson *et al.*, 1984). It is important to note that in quiescent or terminally differentiated cells resting in G_0, mRNAs and the protein levels of both R1 and R2 are not detectable (Björklund *et al.*, 1990; Hengstschläger *et al.*, 1994*a*)

The salvage pathway

The salvage pathway is a complementary route to the *de novo* pathway which provides cells with DNA precursors and also prevent waste of nucleosides. If not phosphorylated these nucleosides will otherwise leak out from the cells and be rapidly degraded. The uptake of deoxyribonucleosides from extracelluler space, derived *in vivo* from nutrients or degraded DNA is a process facilitated by nucleoside carrier proteins, present in almost all animal cells (Plagemann *et al.*, 1988). The transport process is believed to be the first regulatory step in the salvage pathway. The next step is the phosphorylation of deoxyribonucleosides to their corresponding monophosphate form and this step is catalysed by deoxyribonucleoside kinases. This is usually the rate limiting step in the salvage pathway. Deoxyribonucleoside monophosphates will be further phosphorylated to their corresponding triphosphates by nucleotide kinases, thus, completing the salvage cycle. The reuse of free purine and pyrimidine bases is also part of the salvage pathway, but this field

is beyond the scope of this thesis and will not be discussed further. Clinically important nucleoside analogs such as araC (9-β-D-arabinofuranosylcytosine) and araG (9-β-D-arabinofuranosylguanine) can also pass the cell membrane passively due to a higher lipophilicity, but they need to be phosphorylated by deoxyribonucleoside kinases, in order to be active (Plagemann and Woffendin, 1989).

In mammalian cells there are four deoxyribonucleoside kinases, namely cytosolic deoxycytidine kinase (dCK), cytosolic thymidine kinase (TK1), mitochondrial thymidine kinase (TK2) and mitochondrial deoxyguanosine kinase (dGK). Adenosine kinase (AK) may also be considered as a member of this family of enzymes since AK accepts deoxyadenosine as substrate although with low efficiency. Cytosolic 5'-nucleotidases are enzymes that dephosphorylates nucleoside monophosphates to their corresponding nucleosides. However, one of the cytosolic 5'-nucleotidases has also the capacity to phosphorylate nucleosides and nucleoside analogs, such as ddI (Johnson and Fridland, 1989). However, this thesis will focus on the two mitochondrial deoxyribonucleoside kinases: TK2 and dGK. The metabolic pathways of the DNA precursors are schematically illustrated in Fig.1 and a summary of the properties of the deoxyribonucleoside kinases are found in Table 1 (page 30).

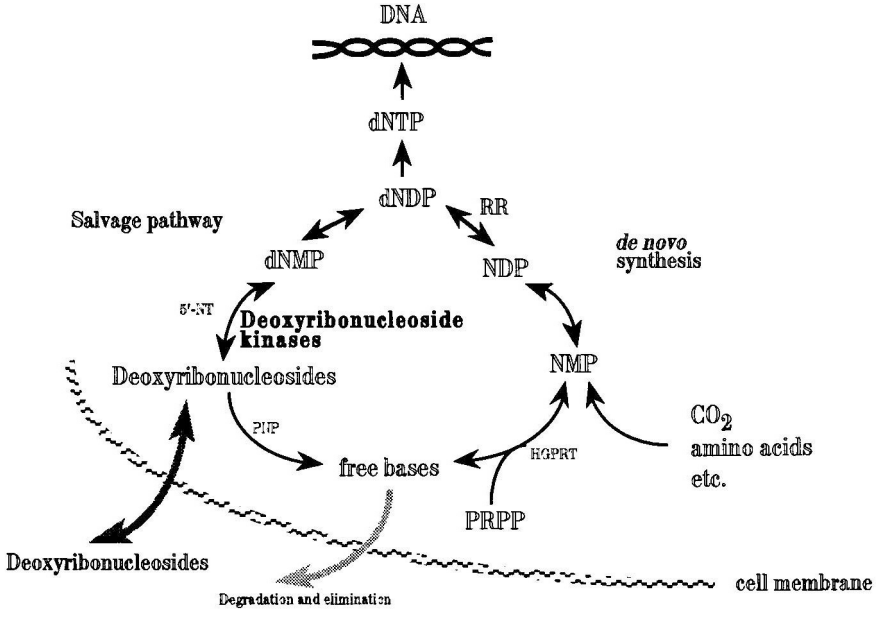


Fig. 1. Simplified metabolic pathways of the DNA precursors. Selected enzymes of both *de novo* and salvage pathways are shown. RR, ribonucleotide reductase; HGPRT, hypoxanthine-guanine-phosphorylribosyl-transferase; PNP, purine nucleoside phosphorylase; 5'-NT, 5'-nucleotidase; Deoxyribonucleoside kinases are in bold letters. The uptake of deoxynucleosides is a process facilitated by nucleoside carrier proteins.

Distribution of dNTP pools

Generally, in proliferating cells deoxynucleotides are synthesised *de novo* in the cytoplasm and then transported into the nucleus or mitochondria to be used for DNA synthesis. Presumably there is a transport system for deoxynucleotide passage from the cytoplasm into the mitochondrial matrix. Studies of *S. cerevisiae* mitochondrial DNA polymerase indicated that high dNTP concentrations stimulates the pol γ activity and the dNTP concentrations may be rate limiting for the replication of mtDNA (Lecrenier and Foury, 1995). Therefor, in S-phase cells there might be a higher mtDNA replicating rate where the *de novo* synthesis is high than that in resting cells where the intracellular dNTP levels are low. However, yeast cell mtDNA synthesis may differ from that of mammalian cells since the dNTP supply for yeast mtDNA synthesis appears to solely depend on the *de novo* synthesis because yeast cells do not contain salvage enzymes. Constantly replicating mtDNA needs dNTP pools irrespect of the cell cycle phase. Thus, the supply of DNA precursors for mtDNA synthesis is differently regulated than that used for nuclear DNA synthesis.

A study by Bogenhagen and Clayton (1976) suggested that there are distinct regulatory mechanisms affecting the precursor pools for nuclear and mitochondrial DNA synthesis, in other words mitochondria contain distinct pools of DNA precursors, different from the pools for nuclear DNA synthesis. This was later confirmed by Bestwick *et al.* (1982), who showed that after treatment of cells by antimetabolites the four mitochondrial dNTP pools actually expanded even though the total cellular pools of dTTP and dGTP were severely depleted. Mitochondrial dNTP pools are larger, in relation to the mtDNA content as compared to the whole cell pools.

It is clear that deoxyribonucleotide pool imbalances may be mutagenic (Kunz, 1988; and Meuth, 1984, 1989) or lethal (Yoshioka *et al.*, 1987), and together with the enzymes of the *de novo* pathway, the enzymes of the salvage pathway strive to maintain balanced dNTP pools. A study of thymidine kinase deficient cells showed a decreased dTTP pool in the mutant cells as compared to wild type cells and the mutant cells were hypersensitive to the cytotoxic and mutagenic effects of DNA alkylating agents (Wakazono *et al.*, 1996). Recent studies demonstrated that interleukin-3 (IL-3) treatment of cells up-regulate the salvage pathway of dNTP synthesis, particularly the thymidine kinase activity. Apparently it is important to control cellular dNTP pools in haemopoietic cells, since withdrawal of IL-3 leads to a rapid imbalance in dNTP pools preceding DNA fragmentation and apoptosis (Oliver *et al.*, 1996).

Basic properties of the deoxyribonucleoside kinases

1. Cytosolic thymidine kinase

Thymidine kinase 1 is present in almost all types of organisms. The expression of TK1 is tightly regulated during the cell cycle, with transcriptional, post-transcriptional, translational, as well as post-translational regulatory mechanisms.

The gene of human TK1

The human TK1 gene was cloned and characterised more than ten years ago (Bradshaw, 1983; Bradshaw and Deininger, 1984; Flemington *et al.*, 1987). TK1 cDNA is 1,421 base paris long and has an open reading frame of 702 bp that encodes a protein of 25.5 kDa. The complete gene spans 12.9 kb with seven exons (Fig. 2). Within the introns 13 repetitive sequences of the *Alu* family are found. Thus, the human TK1 gene has one of the highest densities of the *Alu* repeats in the human genome (Flemington et al., 1987). The TK1 gene has been localized to chromosome 17 (McDougall *et al.*, 1973). The homology of the TK1 cDNA sequence among different species is high, for example, at the amino acid level there is 80% similarity between human and mouse TK1.

Human TK1 gene regulation

The human TK1 gene and its promoter structure is shown in Fig. 2. The TK1 promoter consists of an A-T rich site (TATA box), two inverted CCAAT boxes, multiple SP1 binding sites, an E2F binding site and a cycloheximide-sensitivity response element (Kreidberg and Kelly, 1986; Flemington *et al.*, 1987; Xia *et al.*, 1995).

In normal growing cells, both TK1 mRNA and protein rise near the G1/S boundary, peak in early S phase, and decline in the G2 phase to the levels observed in G1 (Wintersberger *et al.*, 1992; Hengstschläger *et al.*, 1994a). In general, the transcription factor E2F, which promotes expression of the S phase regulated genes, as well as several other protein factors, have been proposed to be essential for the G1/S regulation of the human TK gene (Ogris *et al.*, 1993; Anderson *et al.*, 1996; Hengstschläger *et al.*, 1996). A trans-acting factor, CBP/tk (CCAAT binding protein for TK gene), binds to either one of the two inverted CAATT boxes in the human TK1 gene promoter in a cell cycle and age dependent manner. This factor might modulate the expression of TK1 mRNA. (Chang and Cheng 1993; Pang and Chen, 1993). There is evidence that transcriptional activation of the TK1 promoter is differently regulated in normal and tumor cells. For example over expressed cyclin A or E but not D may act as positive regulators in normal human diploid IMR-90 fibroblasts but not in HeLa cells (Chang and Huang, 1996). In primary cells, the expression of thymidine kinase activity and the mRNA levels are strictly S phase specific. In contrast, DNA tumor virus-transformed

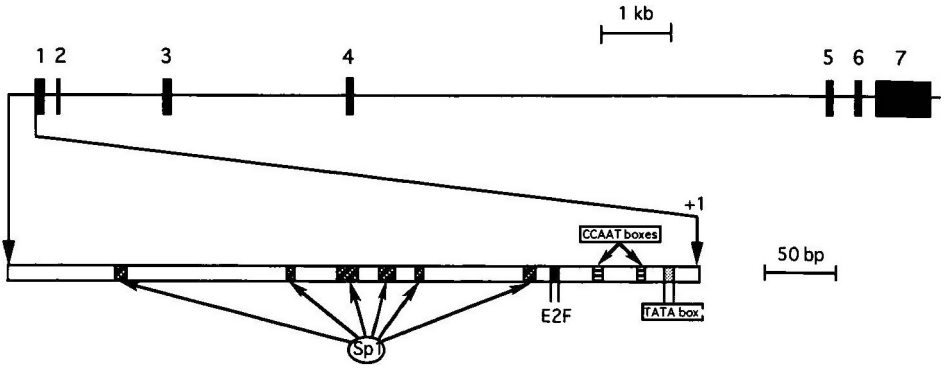


Fig. 2. The structure of the human TK1 gene. The upper line depicts the human TK1 gene, black bars with number indicate the exons; The lower bar represents the 5'-upstream sequence with the promoter elements indicated; +1 is the transcription initiation site.

cells have enhanced and constitutive levels of TK1 mRNA during the whole cell cycle. Their TK1 protein abundance, however, still increases at the G1-S transition and stay high throughout G2 until mitosis. Therefor, post-tran--scriptional control must account for the decoupling of mRNA and protein synthesis in G1. (Stewart *et al.*, 1987; Chang, 1990; Gudas *et al.*, 1993; Mikulits and Mullner, 1994). The underlying mechanism was thought to be dependent on the total amount of TK1 mRNA as suggested by Mikulits *et al* (1996). A S phase specific activator of translation was not compatible with the furhter rise of in mRNA but the exact nature of the regulatory process is not yet known.

During mitosis, the abundance of TK1 polypeptide is rapidly decreased by proteolysis indicating post-translational regulation of TK1 expression (Gudas *et al.*, 1993; Hengstschläger *et al.*, 1994*b*). It was shown that the carboxyl-terminal 40 amino acids of human TK1 were responsible for the M-phase specific degradation of the TK1 polypeptide (Kauffman and Kelly, 1991). The mutations in C-terminal part of the TK1, which abolished the specific degradation at mitosis/G1, also allowed TK1 protein and activity to be expressed in quiescent mouse cells transfected with human TK1 cDNA (Kauffman *et al.*, 1991). The TK1 polypeptide was found to be phos-phorylated in HeLa cells (Chang and Huang, 1993) and several other human cell lines during M-phase (Chang *et al.*, 1994). The phosphorylated TK1 in HeLa cells had 10-fold lower affinity for its substrate. This decrease was proposed to be a mechanism for cells to prevent unnecessary synthesis of dTTP at the time of mitosis.

Properties of human thymidine kinase 1

Active human TK1 has a molecular mass ranging from 55 kDa to 110 kDa, respectively, and a subunit molecular mass of 25.5 kDa. A dimer or a tetramer is formed in the absence or presence of ATP, respectively (Munch-Petersen *et al.*, 1991, 1993).

Purified human TK1 (Munch-Petersen *et al.*, 1995*a*, 1995*b*) revealed an ATP and enzyme concentration dependent activation phenomenon. When thymidine was used as substrate, incubation with ATP at 4°C induces a reversible, enzyme concentration-dependent, kinetically slow transition from a low to a high affinity form. Recombinant human TK1, purified from *E. coli* extracts, displayed only the high affinity form kinetics regardless of a pre-exposure to ATP. TK1 purified from a patient with acute monocytic leukemia was not activated by ATP. The explanation to these differences were probably the absence of post-translational modifications in *E. coli* and altered post-translational modifications in acute monocytic leukemia cells (Jensen and Munch-Petersen, 1994). ATP as well as other nucleoside triphosphates can serve as phosphate donors in the reaction, but dTTP or CTP can not.

In addition to the natural substrates, thymidine and deoxyuridine, TK1 also phosphorylates a number of clinically important nucleoside analogues. For instance the anti-HIV compounds, AZT and FLT (3'-fluoro-2',3'-deoxy-thymidine) (Furman *et al.*, 1986; Matthes *et al.*, 1988). Among the 5-substituted deoxyuridine analogues, 5-fluoro, 5-bromo, and 5-ethyl analogs were accepted but not analogs with bulkier substitutions, such as 5-propenyl, 5(2-chloroethyl) and 5-(2-bromovinyl) deoxyuridine derivatives (Eriksson *et al.*, 1991, 1995).

2. Deoxycytidine kinase

Deoxycytidine kinase is a cytosolic enzyme, that catalyses the phosphorylation of deoxycytidine to deoxycytidine 5'-monophosphate using a nucleoside triphosphate as phosphate donor. Unlike cytosolic thymidine kinase, dCK is not cell cycle regulated, but consecutively expressed in many cells. Earlier studies demonstrated a high dCK activity in resting lymphocytes and a modest (2- to 3-fold) increase of the activity after phytohemagglutinin stimulation. The highest level of dCK was found in lymphoid tissues and the lowest in nerve, liver and muscle tissues.

The gene for human deoxycytidine kinase

Human dCK cDNA was cloned and expressed 5 years ago (Chottiner *et al.*, 1991). The full length human dCK cDNA was 2460 bp long, including a 780 bp ORF encoding a protein with a calculated molecular weight of 30.5 kDa. A conserved nucleotide binding motif (aa 28-34) and a N-linked glycosylation site (aa 148-150) were identified. The human dCK gene has been localized to chromosome 4 band q13.3-q21.1 (Song *et al.*, 1993; Stegmann *et al.*, 1993). The complete gene extends over more than 34 kb and is composed of 7 exons ranging in size from 90 to 1544 bp. The second intron is large (>19 kb), and may play a role in the tissue-specific expression of the gene (Song *et al.*, 1993). The mouse and rat dCK sequences show great similarities to the human one and at the amino acids level they are 93.4% and 91.9% identical, respectively (Karlsson *et al.*, 1994; Stegmann *et al.*, 1994). However, the

mRNA transcripts from the Northern blot analysis showed differences in length in case of these three species, 2.8 kb for human dCK, 3.4 kb for mouse dCK and 4.2 kb for rat dCK.

Human deoxycytidine kinase gene regulation

The regulatory elements, found in the 5'-region of the human dCK gene (Mitchell *et al.*, 1993; Song *et al.*, 1993; Chen *et al.*, 1995), includes a 5'-GC box, an E box, a 3'- GC box, and an E2F binding site. No CCAAT or TATA boxes were found (Fig. 3). Studies of dCK promoter activity using

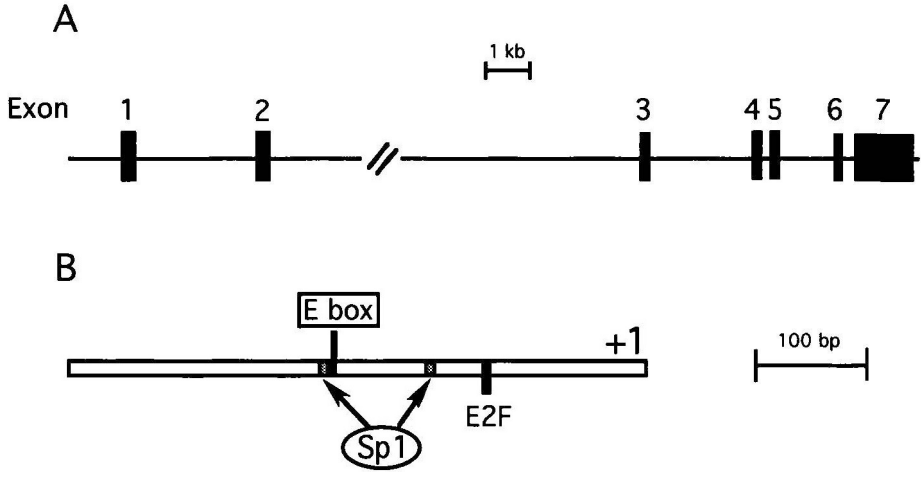


Fig. 3. Schematic presentation of the human dCK gene. A. The gene structure, exons are indicated by black bars; B. The promoter structure, regulatory elements are indicated; +1 refers to the translation start site.

T and B lymphoblast cell lines and nuclear extracts revealed that the transcription factor Sp1 binds to both GC boxes, activating when bound at the 5' site but repressing when bound at the 3' site. Transcription factor E2F binds weakly to the E2F sits *in vitro* and does not appear to mediate cell cycle specific expression of the dCK gene. No significant differences were observed in promoter activity or transcription factor binding between Jurkat T and Raji B lymphoblasts. The conclusion was that the dCK gene is not cell cycle regulated but is regulated by a number of ubiquitously expressed transcription factors. This is in agreement with the study of dCK activity and mRNA levels during the cell cycle, no variation of dCK activity was found during the cell cycle of HL-60 cells and a steady state level of dCK was seen irrespective of the cell cycle phase (Hengstschläger *et al.*, 1994*a*). Obviously, this gene is not a target for the regulation observed with S-phase related enzymes such as TK1.

Based on the differences of dCK mRNA and the activity seen in different cell types a post-transcriptional regulatory mechanism was proposed

(Hengstschläger *et al.*, 1993). In that study, four permanent cell lines were used, the mRNA levels of all the four cell types never displayed any cell cycle dependent changes, but in case of the dCK activities differences were found. Two cell lines (3T6 mouse fibroblasts and human EBV-transformed lymphocytes) did not display any cell cycle regulation of the dCK enzyme activity, whereas in two other cell lines (HeLa cells and COP-8, polyoma-transformed mouse fibroblasts) a 10- and 15-fold induction of dCK enzyme activity at the G1/S boundary of the cell cycle was observed. The half life of the dCK protein in 3T6 cells and EBV-transformed human lymphocytes was longer than that observed in HeLa and COP-8 cells. Thus, the stabilities of the dCK protein may be a regulatory factor in the cell specific expression of this protein.

Properties of deoxycytidine kinase

Active human dCK has an apparent molecular weight of 60 kDa, *i. e.* a dimer of two 30 kDa subunits (Bohman and Eriksson, 1988) in agreement with the cloned human dCK cDNA (Chottiner *et al.*, 1991). Bohman and Eriksson (1988) did not find any dCK isoforms, in contrast to the mitochondrial and cytosolic forms of dCK reported by Cheng (1977) and Wang *et al.* (1993). The former type of dCK is most likely the mitochondrial thymidine kinase (TK2) since it showed similar catalytic activity and substrate preference to that of TK2. However, the subunit molecular mass of mtdCK (Wang *et al.*, 1993) was not in agreement with neither the cloned human dCK nor that of TK2. The reason for this discrepancy is not clear.

Human dCK phosphorylates deoxycytidine, deoxyadenosine, deoxyguanosine and their analogues with complex kinetics. The mechanism of the reaction was determined to be a random Bi Bi , or an ordered sequential pathway by Kim and Ives (1989) and Datta *et al.* (1989). It was later observed that UTP was a better phosphate donor for dCK than ATP but less efficient than a mixture of nucleoside triphosphates (White and Capizzi, 1991; Shewach *et al.*, 1992). This result was confirmed by other studies, and it was concluded that the major intracellular phosphate donor for dCK is not ATP, but most likely UTP (Kraweic *et al.*, 1995; Ruiz van Haperen *et al.*, 1996).

It is clear that purine deoxyribonucleotides show higher Km and Vmax values with dCK than that observed with deoxycytidine. In addition to the natural substrates, dCK phosphorylates numerous analogs with both 2'- and 3'-modifications, as well as acyclic sugars, provided that the base is cytosine with minor substitutions at 5-position. However, 5-substitution with bulky group were not acceptable (Eriksson *et al.*, 1995). With uracil or thymine as base dCK showed very low activity. No purine nucleosides with 3'-modification or acyclic sugars were phosphorylated, but arabinofuranosyl nucleosides were accepted as substrates for dCK (Eriksson *et al.*, 1991, 1995).

3. Mitochondrial thymidine kinase

Thymidine kinase 2 is a mitochondrial enzyme (Berk and Clayton, 1973) which catalyses the transfer of a gamma phosphate group from ATP to the 5'-hydroxyl group of deoxythymidine, the same substrate as for the cytosolic thymidine kinase (TK1). However, these two thymidine kinases are different proteins encoded by two separate genes. The level of TK2 is low as compared to TK1 in proliferating cells, but it is significant in resting or terminal differentiated cells where TK1 activity is undetectable. The expression of TK2 is most likely correlated to the mitochondrial content of the tissues but not to the growth state of the cell. The human TK2 gene has been localised to chromosome 16 by using mouse/human hybrid cell lines (Willecke *et al.*, 1977).

Properties of thymidine kinase 2

A homogenous preparation of TK2 from human leukemic spleen had a subunit molecular mass of 29 kDa. The molecular weight of the active enzyme was estimated to be 30 kDa (Munch-Petersen *et al.*, 1991).

Human TK2 utilises thymidine, deoxycytidine and deoxyuridine as its substrates but with different efficiency and kinetic mechanisms. TK2 phosphorylates thymidine with negative co-operativity, which means that the affinity for the substrate decreased with increasing substrate concentrations. The K_m values for thymidine was 16 μM at thymidine concentrations above 8 μM, and was 0.2-0.4 μM at thymidine concentration below 8 μM. The phosphorylation of deoxycytidine and deoxyuridine showed normal Michaelis-Menten mechanism with K_m value of 36 μM and 6 μM, respectively. Both dTTP and dCTP inhibit the enzyme and ATP and CTP can be used as phosphate donors by TK2 (Munch-Petersen *et al.*, 1991).

Thymidine analogs with modification on the sugar moiety such as 3'-azido-2',3'-deoxythymidine (AZT), arabinofuranosyl thymine (ara-T), 3'-fluoro-2',3'-deoxythymidine and ribothymine could be phosphorylated, but with low efficiency. TK2 showed about 5-10 % of the activity with AZT as compared to that with thymidine. A large number of deoxyuridine analogues can be phosphorylated by TK2 including 5-substitutions such as halogen, amino, ethyl, and some bulky groups as well as arabinosyl uracil (Munch-Petersen *et al.*, 1991; Eriksson *et al.*, 1991, 1995). The phosphorylation of FIAU by TK2 may in part explain the cytotoxic effect of this anti-hepatitis B compound (Wang and Eriksson, 1996). Deoxycytidine analogues with 5-substitution of halogen, amino or even bulky groups, such as 5(2-chloroethyl), 5(2-bromovinyl) showed activity with TK2 and modification of the sugar moiety such as 2'-fluoro was also acceptable. Interestingly some of the 5-aryl substituted deoxycytidine analogues were phosphorylated by TK2 but not by dCK (Eriksson *et al.*, 1995). Results from the study of the substrate properties of TK2 with araT led to a selective assay method for TK2 measurement in crude extracts (Arnér *et al.*, 1992).

4. Deoxyguanosine kinase

Mammalian deoxyguanosine kinase (dGK, NTP:deoxyguanosine 5'-phosphotransferase, EC 2. 7. 1. 113) is a nuclear gene product, localised to the mitochondria, catalysing the phosphorylation of purine deoxyribonucleosides and their analogs, using a nucleoside triphosphate as phosphate donor. The expression of dGK is not cell cycle regulated and the activity of dGK is found in most tissues, including lymphoid tissues, spleen, skin, liver and brain, although in most cases at a low level compared to cytosolic dCK (Yamada *et al.*,1982; Sarup and Fridland, 1987; Park and Ives, 1988; Bohman and Eriksson, 1990; Wang *et al.*, 1993).

Properties of deoxyguanosine kinase

The active dGK protein is a dimer of two 28 kDa subunits. dGuo and dIno were efficiently phosphorylated by dGK, but the specific activity of this enzyme was low in all cases possibly because of inactivation of the enzyme during purification (Park and Ives, 1988; Arnér and Eriksson, 1995).

A random Bi Bi kinetic mechanism, which may include the formation of a dead-end complex, was recently proposed for bovine dGK (Park and Ives, 1995). The nucleoside diphosphate product (ADP or dTDP) exhibited competitive inhibition versus the phosphate donor (ATP or dTTP), but was non-competitive towards the phosphate acceptor. The monophosphate product (dGMP or dIMP) was competitive towards both the phosphate donor and the phosphate acceptor. The end product dGTP behaved as a bisubstrate analog, most likely interacting with its nucleoside moiety towards the dGuo site, and the phosphate groups of dGTP interacting with the ATP binding site. This result indicates that the binding of both phosphate donor and phosphate acceptor occurs at adjacent sites (Park and Ives, 1995). This is most likely a general mechanism for the feed back inhibition of deoxyribonucleoside kinases.

5. Adenosine kinase

Adenosine kinase (AK, EC 2.7.1.20) catalyzes the phosphorylation of adenosine to its 5'-monophosphate using a nucleoside triphosphate or a nucleoside monophosphate as phosphate donor. This enzyme is abundant and is widely distributed among many tissues. Adenosine is the natural substrate, formed either by the dephosphorylation of AMP or the hydrolysis of S-adenosylhomocystein. As one of the two principal enzymes of adenosine metabolism in mammalian cells, adenosine kinase has a critical role in regulation of cellular adenosine concentrations. This enzyme is also involved in the activation of certain cytotoxic purine nucleoside analogs (Arch and Newsholme, 1978).

The gene for adenosine kinase

The cDNA of human AK has been cloned recently (Spychala *et al.*, 1996). The full length cDNA is 1.8 kb long, including a 1035 bp ORF that encodes a

protein of 345 amino acids with a calculated molecular weight of 38.7 kDa. Two polyadenylation signals were found in the 3'-untranslated region of the longest cloned sequence. The alternative usage of polyadenylation sites resulted in two cDNA's with different lengths that match the RNA transcripts seen in Northern blot analysis. The highest expression levels of AK mRNA were found in placenta, liver, muscle and kidney. Heart and pancreas showed intermediate levels. A relative low level of mRNA were found in brain and lung. Alignment of the human AK sequence with the sequences from other species revealed high homology to the chinese hamster, the rat, and the mouse sequences, 91.6%, 90.1% and 86.7%, respectively (GeneBank accession number U26588 (chinese hamster), U57042 (rat) and U26589 (mouse)).

There is no significant homology between AK and other nucleoside kinases, including dCK and TK1, or with other adenosine or adenylate metabolic enzymes such as adenosine deaminase, S-adenosylhomocyterine hydrolase, or AMP deaminase. However, striking similarities were observed with a number of plant and microbial sugar kinase. These results indicate that AK is structurally distinct and belongs to an other enzyme family as compared to deoxyribonucleoside kinase family (Gentry, 1992; Wang *et al.*, 1996b; Spychala *et al.*, 1996).

Properties of adenosine kinase

Adenosine kinase has been purified from a number of sources, including rabbit liver (Miller *et al.*, 1979), human placenta (Andres *et al.*, 1979), liver (Yamada *et al.*, 1981) and tumor cells (Schnebli *et al.*, 1967), rat liver (Ogasawara *et al.*, 1979), heart (Fisher and Newsholme, 1984) and protozoan parasite L. *donovani* (Datta *et al.*, 1987). AKs purified from mammalian source are similar, but differences among species were also observed (Yamada *et al.*, 1982).

Active human AK is a monomer of 37.25 kDa. The preferred phosphate donors are ATP and GTP. Other nucleoside triphosphates, *e.g.* dATP, dGTP can be used as phosphate donor but with lower efficiency. A divalent cation is required for catalysis. Mg^{2+} is preferred but other cation such as Mn^{2+} and Co^{2+} can also be used. High concentrations of adenosine, Mg^{2+} and ATP are inhibitory for the mammalian enzymes.

The reaction mechanism was found to be of the ordered Bi Bi type where adenosine is the first substrate to bind the enzyme, and AMP is the last product to dissociate from the enzyme (Palella *et al.*, 1980; Rotllan and Portugal, 1985; Hawkins and Bagnara, 1987). It was suggested that adenosine kinase has at least two adenosine binding sites, one at the catalytic center and another non-productive site at which binding of adenosine protects a thiol group necessary for activity (Hawkins and Bagnara, 1987).

In addition to adenosine AK could also phosphorylate a number adenosine analogues. The enzyme was relatively nonspecific with regard to the base

moiety of ribonucleosides. The best substrates were adenosine analogs, such as 8-aza-adenosine, 2-chloroadenosine, toyocamycin, and sangivamycin. A variety of other purine and purine ribonucleoside analogues were phosphorylated but with decreased substrate efficiency. None of the pyrimidine ribonucleosides tested were substrates or inhibitors. The enzyme was relatively specific for the sugar moiety. 2'-deoxyadenosine (K_m 250 μM) and arabinosyladenine (1 mM) were poor substrates as compared to adenosine (Miller *et al.*, 1979). However, the relatively low level of phosphorylation of dAdo and dAdo analogs by AK is still be significant in cellular metabolism, since the AK levels are much higher than those of other purine deoxyribonucleoside kinase such as dCK (Snyder and Lukey, 1982).

Except for the kinase activity described above, AK purified from rat liver was found to catalyze the exchange reaction between adenosine and AMP in the absence of ATP (Bontemps *et al.*, 1993). ADP had a stimulatory effect on this reaction (Mimouni *et al.*, 1994). A variety of mononucleotides could also act as phosphate donors (Sayos *et al.*, 1994), and a number of purine nucleoside analogs could be used as phosphate acceptors, for instance, 2',3'-dideoxyguanosine (ddG) is phosphorylated by AK in duck hepatocytes in this exchange reaction (Kitos and Tyrrell, 1995).

AK was found in roughly equivalent levels in human liver, kidney, pancreas and cortex, but low in the lung (Snyder and Lukey, 1982).

6. Properties of herpes virus thymidine kinases

Most herpes viruses encode thymidine kinase genes which are considered to be virulence factors. Herpesvirus TKs, like other TKs, catalyses the formation of thymidylate from thymidine using a nucleoside triphosphate as phosphate donor. Herpesvirus TKs, differ from cytosolic TKs but are relatively similar to the mitochondrial TK (see section 3), can utilise a broad range of substrates in addition to thymidine, including deoxycytidine and a large number of nucleoside analogs such as the antiherpes compound acyclovir (ACV) and ganciclovir (GCV). For example, HSV-1 TK has high affinity for thymidine (K_m 0.5 μM), whereas the K_m values for ACV and GCV are much higher. Both ATP and CTP can be used as phosphate donor, and HSV TKs are less sensitive to feed back inhibition by dTTP. An ability that is unique to most herpesviral TKs is the phosphorylation of nucleoside monophosphate to its diphosphate form. This is a property which is essential for the sensitivity and selectivity of several antiviral drugs. For example, herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV), which are sensitive to BvdU, expressed TKs that phosphorylates this compound to both mono- and diphosphate forms, whereas HSV-2, which is resistant to BvdU, expressed a TK that phosphorylates BvdU to its monophosphate form only (Gentry, 1992).

Present investigation

The aim of the present investigation was to characterize the mitochondrial deoxyguanosine kinase and mitochondrial thymidine kinase both at the enzyme and gene level. In order to do that I decided to clone the cDNA of these enzymes and express the proteins in *E. coli.*

Molecular characterization of mitochondrial deoxyguanosine kinase (paper I, III)

The deoxyguanosine kinase protein

In order to clone the dGK gene the enzyme was purified from bovine tissues. The level of dGK activity was measured in the crude extracts of human and bovine tissues, including human brain, spleen, skin, and bovine brain. It was found that brain contains 10-fold higher dGK activity than the other tissues. Therefor, bovine brain was chosen as starting material for dGK purification. By means of DEAE chromatography, hydroxylapatite chromatography and dGMP-sepharose affinity chromatography, dGK was purified 20,000-fold to apparent homogeneity. The native bovine dGK is a dimer with a molecular weight of 56 kDa (subunit molecular mass of 28 kDa) as judged by gelfiltration chromatography and SDS gel electrophoresis. This is the first report of a purification and characterization of dGK from brain tissue.

Earlier published results from dGK preparations showed that dGK only phosphorylated dGuo and dIno. In this work we clearly show that dGK is able to phosphorylate all the three natural occurring purine deoxynucleosides, dAdo, dGuo and dIno. The presence of ATP greatly stabilised the enzyme but storage in phosphate buffers abolished the capacity of the enzyme to phosphorylate dAdo and other purine nucleoside analogs.

dGK phosphorylates dGuo (K_m 7.6 μM), dAdo (K_m 60 μM and V_{max} 50% of that of dGuo) and dIno. dCyd was shown to be phosphorylated by dGK at high concentration (200 μM) (Paper III). However high concentration of dCyd (1 mM) produced no inhibition of the phosphorylation of the other dGK substrates.

In addition to the natural substrates, dGK phosphorylates a number of nucleoside analogs, including araG (K_m 8 μM and a V_{max} 50% of that of dGuo), CdA (K_m 85 μM and a V_{max} similar to that of dGuo) and araHx (3-fold better phosphorylation than dGuo). AraA, 7-deaza-2'-dAdo, several 2'-modified Guo analogs and carbocyclic dGuo were also phosphorylated. Several 3'-modified purine 2'-deoxyribonucleoside analogs were tested, but none of them were phosphorylated; neither were the acyclic guanosine

analogs nor 6-substituted arabinosyl nucleosides. This indicated that the 3'-hydroxyl group of the deoxyribose moiety is a prerequisite for dGK activity and it is probably involved in the binding of substrates.

CdA is clinically used in the treatment of leukemias. It is was generally thought that dCK was the only enzyme responsible for its activation. However the present work clearly shows that dGK phosphorylates CdA as well.

araG, a selective agent against T cell malignancies, is also a good substrate for dGK. Earlier study showed that the level of PNP is similar in both T and B cells, while the level of dGK is higher in T cell than in B cell. araG is selectively toxic to T cells and this may be due to the accumulation of araGTP, demonstrating a role for dGK in araG cytotoxicity.

In tissues that do not express dCK or express dCK at very low level, such as brain, phosphorylation of CdA and araG by dGK is of importance for the evaluation of the cytotoxic effects of these compounds.

The human deoxyguanosine kinase gene

The human dGK cDNA has been cloned recently by two groups (Paper III; Johansson and Karlsson, 1996). The human dGK cDNA consists of a 780 bp ORF encoding 260 amino acids and both a 5'-and a 3'-untranslated region, in the 3'-untranslated sequence a polyadenylation signal and a poly A tail was found. Northern blot analysis showed a tissue specific pattern of expression of dGK mRNA. There are apparently two transcripts of dGK expressed, a major transcript (1.35 kb) and a minor transcript (1.0 kb). High levels of transcription were found in testis, ovary, thymus, liver and brain and lower levels were found in kidney, placenta, colon and peripheral blood leukocytes. The shorter transcripts varies from tissue to tissue. In brain the 1.0 kb transcript was barely detectable which may indicate that the functional transcript would be the longer one since it correlates better to the levels of dGK activity measured earlier. The deduce amino acids sequence of dGK showed 46% identity to that of cytosolic dCK and 21.5% identity to the sequence of mitochondrial thymidine kinase. The expression of dGK cDNA in pET system yielded an active protein with identical catalytic activity to the purified bovine dGK.

Alignment of the human dGK sequence with that of dGK subunit of the heterodimeric dGK/dAK of *Lactobacillus acidophilus* R26 (Ma *et al.*, 1995) and dGK sequence of *Mycoplasma genitalium* (GeneBank accession no. U39705) (Fig. 4), showed limited homology. The human enzyme has 12.9% and 13.2% homology to the *Lactobacillus* and the *Mycoplasma* enzyme, respectively. However, significant conservation of functional important sequence motifs were identified. The ATP binding glycine loop, and the D/ERS motif that presumably is the substrate binding site and as well as an arginine rich motif were found. Site-directed mutagenesis of the *Lactobacillus*

enzyme showed that D78E, D78A and D78N mutations of dGK subunit yielded an inactive enzyme while a R79K mutant enzyme of dGK subunit had altered kinetic behaviour (Hong *et al.*, 1995). A more detailed discussion of these result will be followed in the general discussion.

The translation initiation site differs in the two publications, but in both cases a functional dGK protein could be expressed, which had similar catalytic activity to that of the native enzyme. It is likely that the N-terminal sequence of the dGK protein is part of the mitochondrial sorting signal. The presumed site for mitochondrial internal peptidase cleavage were in both cases the same. A way to determine the structure of the presequence is to define the gene promoter sequence and identify the translation initiation site.

Fig. 4. Alignment of dGK amino acid sequences of human, L. *acidophilus* R26 and *Mycoplasma genitalium*. Identical residues are in boxes. Conserved sequence motifs are marked with asterisk.

CdA and araG phosphorylation by dGK in crude extracts of malignant human brain tissue (Paper II)

The extensive characterization of the substrate specificity of dGK enabled us to develop a selective assay method for the measurement of the dGK activity in crude extracts. dGuo, dAdo and dIno are the natural substrates for dGK. However, the metabolic pathways for these deoxynucleosides are different.

dGuo is the substrate for both dCK and dGK as well as for PNP. dAdo is the substrate for both dCK and dGK. dIno and dAdo are also substrates for ADA. In crude extracts where all these activities are present, nucleoside analogs that are resistant to PNP or ADA and are specifically phosphorylated by dGK will give the best estimation of the dGK enzyme level. Therefor, araG serves as the most selective and specific substrate for dGK.

Both CdA and araG are clinically important nucleoside analogs. The use of these compounds in the treatment of leukemia is well documented (Carson *et al.*, 1982; Saven *et al.*, 1992). In normal brain tissue dCK activity is undetectable, but the dGK activity is relatively high (Paper I). We wished to determine the CdA and araG phosphorylation in extracts of malignant human brain tumors and to establish the role of dCK and dGK in araG and CdA activation. The results showed high levels of dGK in one extract from an astrocytoma tumor and in two extracts from oligodendroglioma tumors, while in the other extracts from the malignant tumors dGK activities were similar to that in normal brain extracts. Most of the CdA phosphorylation observed was due to the action of dCK, demonstrating an induction of dCK expression in brain tumor cells. The results showed that brain tumor cells are able to phosphorylate both CdA and araG, which may be important for the future use of these analogs in chemotherapy.

Molecular characterization of mitochondrial thymidine kinase (Paper IV)

The gene for human thymidine kinase 2

The human TK2 gene has since long been localized to chromosome 16 by the use of mouse/human hybrid cells (Willecke *et al.*, 1977). The human TK2 cDNA was cloned by using amino acid sequence information from purified human brain and bovine brain TK2. The cloned TK2 cDNA contains an open reading frame of 699 bp, coding for 232 amino acids, and 1264 bp of 3'-untranslated sequence including a polyadenylation signal followed by a polyA tail. At present the very 5'- end of human TK2 cDNA has not been identified. However an *Alu* repetitive sequence was found at the 3'-untranslated region as well as in the 5'- region of one of the cDNA clones. The homology of the TK2 sequence to TK1 is low, about 12% at the amino acid level, while the TK2 sequence shows 40% homology to dCK sequence and 29% homology to dGK sequence. This TK2 sequence is the first report of the cloning of a mitochondrial thymidine kinase gene.

The TK2 gene showed a complex transcription pattern as determined by Northern blot analysis using TK2 cDNA as probe. Multiple transcripts exist in most tissues with two major bands of 4.0 kb and 2.2 kb. In certain tissues there is a longer transcript of about 5.0 kb and a shorter transcript of about 1.35 kb. The overall expression of human TK2 mRNA is highest in ovary, testis

and liver and lowest in colon and peripheral blood leukocytes. The existence of multiple transcripts of TK2 mRNA maybe due to the alternative usage of an additional 3´-polyadenylation signal or to alternatively spliced forms of the TK2 mRNA. The over all pattern of TK2 mRNA expression is in accordance with the enzyme activity levels measured earlier (Arnér and Eriksson, 1995; Munch-Petersen *et al.*, 1991).

Characterization of human recombinant TK2

Recombinant human TK2 (N-terminal truncated), as well as the enzyme from bovine brain, phosphorylates both thymidine and deoxycytidine, with thymidine as a preferred substrate. Thymidine was phosphorylated with negative cooperativity while deoxycytidine phosphorylation followed Michaelis-Menten kinetics. Recombinant human TK2 showed similar capacity to phosphorylate a number of pyrimidine nucleoside analogs. Pyrimidine deoxynucleosides (Thd, dUrd and dCyd), pyrimidine arabinosides (araT, araU, araC) and their analogs were phosphorylated. AraC was phosphorylated by both bovine brain TK2 and recombinant human TK2, which differs from earlier results with purified human TK2 (Munch-Petersen *et al.*, 1991). The results showed that recombinant TK2 have enzymatic properties identical to that of purified bovine or human TK2.

An ATP induced TK2 subunit interaction was observed which needs to be investigated further. The presence of N-terminal histidine tag does not affect enzyme activity, but this his-tagged enzyme has a tendency to form large protein aggregates.

Conclusions

In the present investigation, bovine brain dGK and TK2 were purified to apparent homogeneity. This was the basis of later studies of the substrate specificity and kinetic properties of these enzymes and was also a prerequisite for the molecular cloning of dGK and TK2 cDNAs. The cloning and expression of both dGK and TK2 cDNA and partial characterization of the recombinant enzymes are described. These results enable future detailed characterization of the recombinant enzymes and may clarify their physiological roles in the mtDNA precursor synthesis. Some general conclusions from this thesis can be summarised as follows:

- dGK has a broad substrate specificity; phosphorylating all the natural occurring purine deoxynucleosides and a number of nucleoside analogs, including CdA and araG. This may have important implication for chemotherapy of nucleoside analogs.
- dGK can be selectively measured in crude extracts using araG as substrate.
- dGK is constitutively expressed in all tissues and probably correlated to the mitochondria content of the tissue.
- dGK levels are elevated in certain brain tumors and the CdA and araG phosphorylation in brain tumors may be important in the treatment of brain tumors with nucleoside analogs.
- dGK mRNA expression in different tissues correlates with the enzyme levels in the same tissue.
- Purified recombinant dGK, expressed in *E. coli* have similar properties as the native enzyme purified from tissues.
- the dGK cDNA sequence shows homology to the dCK, TK2 and HSV TK sequence.
- TK2 mRNA showed complex pattern of expression, which appeared to correlate with the enzyme activity levels.
- recombinant human TK2 protein expressed in *E. coli* shows identical catalytic activity with that of earlier purified human TK2
- recombinant human TK2 can use araC as a substrate
- recombinant human TK2 shows subunits interaction as a result of the presence of ATP.
- the TK2 cDNA sequence shows homology to the dCK, dGK and HSV TK sequence.

Table 1. *Basic properties of the deoxynucleoside kinases*

	TK1	TK2	dCK	dGK	HSV-1 TK	AK
EC number	EC 2.7.1.21	No separate EC number	EC 2.7.1.74	EC 2.7.1.113	EC 2.7.1.21	EC 2.7.1.20
Gene	Chromosome 17 cDNA cloned 1983	Chromosome 16 cDNA cloned 1996	Chromosome 4 cDNA cloned 1991	Chromosomal location n.d.* cDNA cloned 1996	Genome has been sequenced	Chromosomal location n.d. cDNA cloned 1996
Protein	96-110 kDa tetramer or dimer 2-4 x 25.5 kDa	30 kDa monomer 29 kDa	60 kDa dimer 2 x 30 kDa	56 kDa dimer 2 x 28 kDa	80 kDa dimer 2 x 40.9 kDa	40 kDa monomer 37.2 kDa
Substrate specificity	Thd, dUrd AZT, FdUrd Base: T, U (5-substitutions) Sugar: 3'-substitutions (H, halogen, N3)	Thd, dUrd, dCyd araT, araC Base: T, U, C (5-substitutions) Sugar: 2'-, 3'-, or arabinofuranosyl sugars.	dCyd, dAdo, dGuo CdA, ddC, araC Base: C (5-halogen), A, G (2-chloro) Sugar: 2'-, 3'-substitutions, acyclic, didehydro (with C as base)	dGuo, dAdo, dIno araG, CdA Base: A, G, Hx (2-halongen) Sugar: 2'-substitutions, arabinofuranosyl	Thd, dCyd ACV, GCV Base: T, U, C, G Sugar: 2'-, 3'-substitutions, arabinofuranosyl, acyclic sugar	Ado, dAdo ddG, ribavirin Base: A allow substitutions Sugar: ribose (deoxyribose and arabinofuranosyl sugars are poor substrates)
Phosphate donors	ATP	ATP, CTP	ATP, UTP	ATP	ATP, CTP	ATP, AMP
Inhibitors	dTTP	dTTP, dCTP	dCTP	dGTP	dTTP	AMP, ADP
Cell cycle regulation	Strictly S phase correlated	Constitutively expressed	Constitutively expressed	Constitutively expressed	Consitutively expressed	Constitutively expressed

*n.d. not determined.

General discussions

The present investigation clarified the role of dGK in activation of certain nucleoside analogs used in chemotherapy such as CdA and araG. The cloning and expression of both dGK and TK2 cDNA established an enzyme family of mammalian source that are structurally and functionally related. Based on the sequence conservation of deoxyribonucleoside kinases and the 3-D structure of HSV1 TK, structur function relationships of mammalian deoxyribo-nucleoside kinases were identified. Knowledge of the properties and regulation of these enzymes will be important for the understanding of the cellular metabolism of DNA precursors and for the design of new drugs that may have better effects and selectivity. Both dGK and TK2 are mitochondrial proteins which are encoded by nuclear genes. The mitochondrial origin of these two enzymes are discussed.

The role of deoxyribonucleoside kinases in chemotherapy

Cellular metabolism of DNA precursors

The properties of the cellular deoxyribonucleoside kinases and HSV TK are summarised in Table 1.

As shown in Fig. 5, salvage of deoxynucleosides is co-ordinated by the combination of phosphorylation and dephosphorylation of deoxynucleosides catalysed by deoxyribo-nucleoside kinases and 5'-nucleotidases, respectively. In the absence of *de novo* synthesis, deoxynucleotides for cellular DNA repair and mtDNA synthesis probably arise via the salvage pathway. It has been shown that survival of monocytes and macrophages depends on DNA repair. dCTP formed via the salvage pathway play an important role in DNA repair system in these cells. Monocytes and macrophages have very low dCTP pools but relatively high dCK activities, exogenous deoxycytidine could therefor partially protect monocytes from DNA replication inhibitors such as aphidicolin (Terai *et al.*, 1991).

At physiological thymidine concentration TK2 shows a high affinity towards thymidine. However, if the thymidine concentration increases the affinity will decrease, and high dTTP concentration will inhibit the enzyme which may enable the cell to maintain a balanced dTTP pool.

In certain tissues such as brain and liver where cytosolic dCK activity is not expressed or expressed at a very low level. dGK is the main purine deoxynucleosides phosphorylating enzyme. Together with TK2, these two enzymes would be able to provide cells with all deoxynucleotide pools needed for mtDNA synthesis as well as for cellular DNA repair processes.

The level of dGK was shown to be significantly higher in T-cell than that in B-cells while purine nucleoside phosphorylase (PNP) activity was similar in

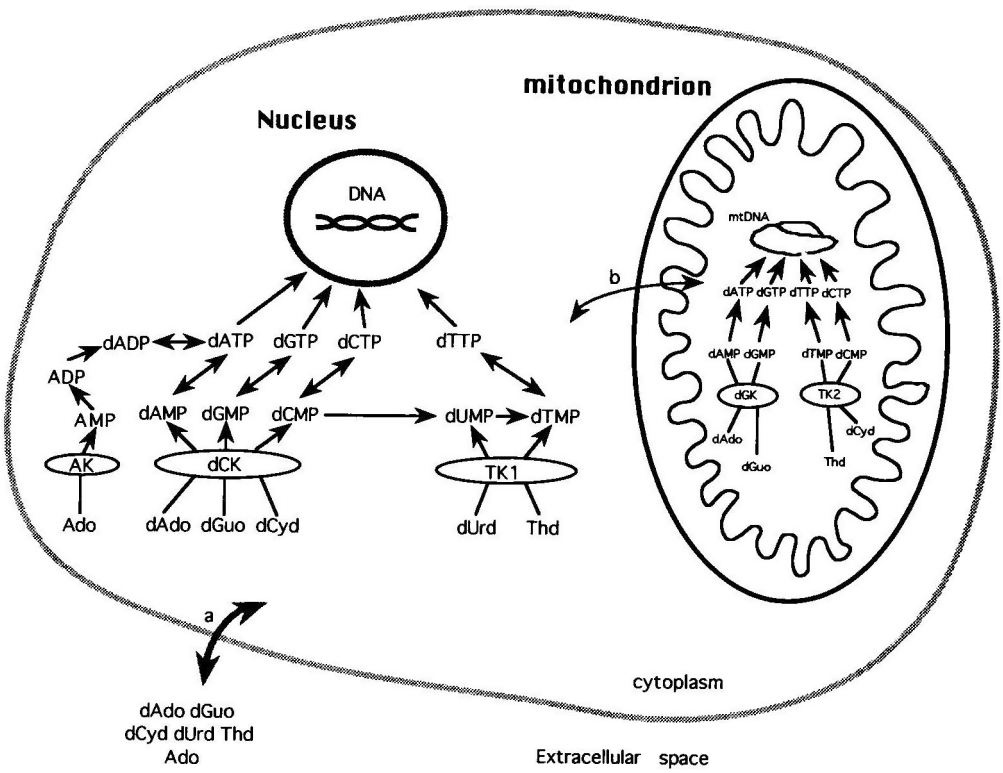


Fig.5. The salvage pathway for deoxynucleosides. The reversible transport of deoxynucleosides (a) and the exchange of deoxynucleotides between mitochondria and cytoplasm (b) is indicated by double arrow bar. Two steps of phosphorylation of deoxyribonucleoside monophosphate to their corresponding triphosphates are simplified by one arrow bar. The action of 5'-nucleotidase is not shown.

both cell types (Osborne and Scott, 1983). PNP deficiency is associated with defective T-cell and normal B-cell immunity. One form of severe combined immunodeficiency disease in man is associated with PNP deficiency. Increased salvage of purine deoxyribonucleoside leading to the accumulation of toxic levels of dGTP in lymphocytes is the assumed mechanism of the disease. The survival of mutant mouse strain which lack PNP activity was found to be due to a secondary loss of dGK activity (Snyder *et al.*, 1994). The latter deficiency most likely protected the PNP mutant mice from the toxicity of dGuo.

Adenosine is toxic to mammalian and bacterial cells; and its presence is associated with inhibition of the immune response, coronary vasodilatation, neurotransmission, and inhibition or stimulation of hormone secretion. Adenosine kinase, together with adenosine deaminase (ADA) and 5'-nucleotidase strive to keep balanced intracellular as well as extracellular adenosine pools. Decreased levels of AK has been associated with resistance to the antiproliferative effects of a number of adenosine analogs (Caldwell *et al.*, 1967), increased purine excretion (Chan *et al.*, 1973) and primary gout

(Nishizawa *et al.*, 1976). In severe combined immunodeficiency syndrome associated with ADA deficiency, the accumulation of phosphorylated derivatives of adenosine and 2'-deoxyadenosine has been implicated as the toxic mechanism (Cohen *et al.*, 1978).

Activation of nucleoside analogs

Clinically used antiviral or cytostatic nucleoside analogs must be phosphorylated in order to achieve their therapeutic effects.

dCK is a key enzyme in the activation of a number of clinically used nucleoside analogues, such as CdA, araC, and araG. Loss of dCK activity or deficiency in dCK expression is one of the mechanisms causing drug resistance, *e. g.* in case of araC, dFdC and ddC resistance (Owens *et al.*, 1992; Ruiz van Haperen *et al.*, 1994, 1995). The molecular basis of the drug resistance was correlated to deletion or specific point mutations in the dCK gene. In case of the araC resistance cell line, two mutation were found; a 115 bp deletion in the coding region and a point mutation Gly33Glu within the ATP binding site, in both case this resulted in completely loss of dCK activity. ddC resistance was the result of a point mutation Gln156Arg reducing the enzyme levels to 10-30% of the wild type activity. Based on the presence of a single point mutation and a marked reduction of dCK mRNA in this cell line, it was postulated that the second allele either is not expressed or is expressed at very low levels (Owens *et al.*, 1992). In the clinical situation mutations of the dCK gene resulting in inactive dCK is a reason but not a frequent reason for araC resistance (Flasshove *et al.*, 1994).

AraG is a substrate for both dCK and dGK, but not for PNP. Unlike AraC, which is a good substrate for dCK and is equally toxic to B and T cells, araG is selectively toxic to T cells (Hebert *et al.*, 1991; Shewach and Mitchell, 1989). The reason for the selectivity relies on the fact that T cells accumulate higher levels of araGTP than other cells, which was recently shown in a preliminary clinical study (Kurtzberg, 1993; Kurtzberg *et al.*, 1995). 2-amino-6-methoxypurine arabinoside is a prodrug of araG with improved solubility, it maintains the selectivity against T-cell tumors, but it is not a substrate for dCK, adenosine kinase, or purine nucleoside phosphorylase, and it is phosphorylated by dGK. A phase I trial of 2-amino-6-methoxypurine arabinoside against haematological malignancies is now in progress (Lambe *et al.*, 1995). Therefor increased knowledge about the properties and regulation of dGK may have large clinical relevance for future treatment of haematological malignancies.

Future prospectives — gene therapy with deoxyribonucleoside kinase cDNAs

The fact that cancer cells usually do not exhibit unique biochemical properties that distinguish them from normal cells is a limitation for cancer chemo-

therapy. There are few drugs that selectively kill tumor cells without affecting the normal cells. Gene therapy offers a prospect of selectively introducing genes into cancer cells, rendering them susceptible to specific anti-metabolites. Herpes simplex type 1 thymidine kinase (HSV1 TK) is a particularly attractive gene in therapeutic approaches. In tumor cell lines transfected with HSV-1 TK gene construct (retroviral vector), the sensitive to the toxic effects of nucleoside such as ganciclovir was found to be dramatically increased, and selective killing of the tumor cells was achieved in cultured cells and in animal models (Ezzeddine *et al.*, 1991; Chen *et al.*, 1994; Wills *et al.*, 1995).

Similar experiments have recently been done with dCK cDNA. Transfection of dCK cDNA into an araC and 5-aza-2'-deoxycytidine resistant rat leukemia cells fully restored drug sensitivity to these cells (Stegmann *et al.*, 1995). Using retroviral and adenoviral vector-mediated transduction of dCK cDNA a marked sensitization of a glioma cell lines to the cytotoxic effects of araC *in vitro* was found. A significant antitumor effects *in vivo* was also observed (Manome *et al.*, 1996). The study of the tumor cell lines transfected with dCK cDNA demonstrated a direct relationship between dCK activity and drug sensitivity. Many solid tumors have relatively low levels of dCK, and it may be possible that dCK gene transfer could be a useful adjunct to chemotherapy in antitumor treatment (Hapke *et al.*, 1996). In the future, engineered human deoxyribonucleoside kinase genes with gene products with improved drug selectivity may be useful in anti-cancer gene therapy.

Structure function relationships of deoxyribonucleoside kinases

Herpes virus TKs show similar characteristics to those of the host enzymes, but they still have important differences in substrate specificity which is the basis for successful antiviral chemotherapy. Today a large number of thymidine kinase genes has been sequenced from various sources, including vertebrates, bacteria, viruses. In the review of Gentry (1992), TKs has been classified into two main groups: the poxviral and cellular thymidine kinases, and herpesviral thymidine kinase and eukaryotic deoxycytidine kinases. The sequence of the mitochondrial thymidine kinase was not known at that time, but it is clear now that the mitochondrial TK is a member of herpesviral TK and cellular dCK family because of their similar biochemical characteristics and gene sequences (Wang *et al.*, 1996a (Paper III)). Mitochondrial dGK is certainly a member of dCK family as well (Wang *et al.*, 1996*b* (Paper IV); Johansson and Karlsson, 1996). The recent release of the 3-D structure of HSV-1 TK (Wild *et al.*, 1995; Brown *et al.*, 1995) and the identification of conserved sequence motifs among deoxyribonucleoside kinases enable a reasonable prediction of structure function relationships among deoxyribonucleoside kinases.

The structure of HSV1 TK

Comparisons of herpesviral TK sequences with that of adenylate kinases have revealed several highly conserved sequence motifs that have been grouped into six sites (Balasubramaniam *et al.*, 1990). Correlation of these sites with the 3-D structure of HSV-1 TK is illuminating. Site 1, a crucial part of the active centre consists of a glycine loop with the sequence fingerprint -G-X-X-G-X-G-K-T-, which is also conserved in the adenylate kinase and numerous ATP or GTP binding proteins. This is the ATP binding domain and the lysine residue is essential for phosphoryl transfer during catalysis. Site 2 is involved in the binding of the nucleoside substrate, particularly residue E^{83} and W^{88}. Site 3 and 4 are at the bottom of the active site as part of the substrate binding site and evidently determine the substrate specificity. Residues D^{162}, R^{163}, Y^{172}, P^{173}, and R^{176} are involved in the interaction with the thymidine base. Residues that include and extend beyond site 2 are in close contact with the sugar moiety of the nucleoside, especially M^{128} and Y^{132}. Site-directed mutagenesis studies of the aspartic acid residue (D^{162}) of the -DRH- motif suggested that D^{162} participates in nucleoside binding but is not involved in ATP or Mg^{2+} binding. The results also indicate that the binding of thymidylate may share or overlap with that of thymidine (Black *et al.*, 1996*b*). Other studies also indicated that amino acids residues that surround the conserved -DRH- motif are important for the substrate specificity of the enzyme. Substitution of $T^{136}I^{137}$ with $L^{136}L^{137}$ in the substrate binding site of the VZV TK yielded a mutant enzyme which lost deoxycytidine phosphorylating activity and had reduced TK and TMPK (thymidylate kinase) activities (Suzutani *et al.*, 1993). Random mutagenesis of the substrate binding site of HSV-1 TK, $L^{159}I^{160}F^{161}$-DRHPIA-$A^{168}L^{169}L^{170}$ led to a number of mutant enzymes with altered substrate specificity, for example the enzyme of mutant $L^{159}\mathbf{L}^{160}\mathbf{L}^{161}$-DRHPIA-$\mathbf{V}^{168}\mathbf{M}^{169}L^{170}$ (mutated residues are in bold letters) has reduced thymidine and ganciclovir phosphorylating activity but 5-fold higher acyclovir phosphorylation than that of the wild type enzyme (Black *et al.*, 1996*a*). Site 5, the arginine rich motif, is similar to a region of adenylate kinase and other nucleotide binding protein and R^{222} is involved in phosphoryl group binding.

Prediction of structure function relationships of human deoxyribonucleoside kinases

Upon the publication of human dCK sequence, a comparison of herpesviral TKs with cellular dCK sequence was made and revealed a clear relationship. Therefor, Harrison *et al.* (1991) proposed that the TKs of herpesviruses of higher and lower vertebrates have evolved either independently or successively from a cellular dCK. The finding of great similarity of dCK sequence to the two newly cloned human mitochondrial deoxyguanosine kinase and human mitochondrial thymidine kinase, thus established a new family of deoxy-ribonucleoside kinases. The sequence of human mitochondrial thymidine

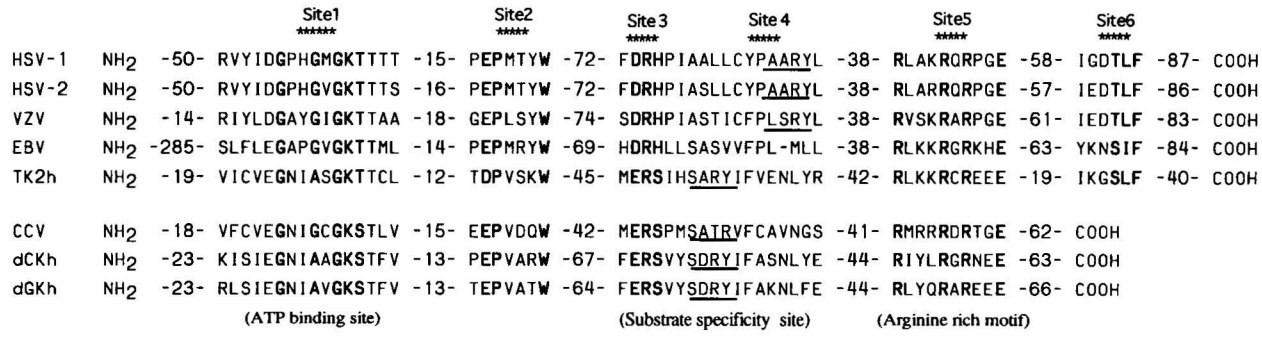
kinase showed significant homology to the eukaryotic dCKs and dGK, as well as to the family of herpesviral TKs, but not to the family of bacterial, poxvrial and eukaryotic cytosolic TKs. The high homology of TK2 to dCKs, dGK and herpesviral TKs suggests that they are evolutionary related, and may originate from a common ancestor. The mammalian deoxyribonucleoside kinases form a family of enzymes which are similar in size, are active as dimers and have broad substrate specificity and phosphate donor capacities, similar to the herpesviral TK family.

Alignment of the human deoxyribonucleoside kinases with selected herpesviral TKs (Fig. 6A) revealed a striking conservation of functional important sequence motifs. An overview of the overall arrangement of these sequence motifs along the entire sequences is shown in Fig.7, suggesting that they are structurally and functionally related, but still the mammalian enzymes have an additional conserved feature that distinguish them from the herpesviral TKs. They contain a leucine zipper type of structure close to the C-terminal of the polypeptide which could also be found in several protein kinases such as CDK activating kinase and cdc protein kinase (Fig. 6B). This domain identified for the first time here may be involved in protein-protein interactions.

The ATP binding site (site 1 in Fig. 6A) close to the N-terminal has a conserved sequence fingerprint -G-X-X-G-X-G-K-T/S-T-, with the exception of EBV TK. This motif is also found in adenylate kinase and a large number ATP/GTP binding proteins. The presumed loop structure facilitates interactions between the γ phosphoryl group of the nucleotide and a positively charged amino acid residue (lysine), a crucial part of the active centre of all kinases as demonstrated by the AK and HSV1-TK structural studies.

The substrate specificity site (sites 3 and 4), a β strand structure including residue 161-192 in HSV1-TK with a conserved sequence triplet -DRH- in all the herpesviral TKs and other thymidylate kinases, has been shown to be important in substrate binding. In the mammalian deoxynucleoside kinases there is a similar triplet -E-R-S-, possibly with the same function. Channel catfish virus (CCV), a lower vertebrate herpes virus, encodes a TK which can be distinguished biochemically from cellular thymidine kinase (TK1) and other herpes virus TKs because of the inability to use CTP as phosphate donor (Harrison *et al.*, 1991). It is of interest to note that CCV TK does not contain the consensus -DRH- motif but instead has the -ERS- triplet which is found in the mammalian dCK family. Another triplet -A/DRY- conserved in all these sequences, with the exception of EBV TK, play an important role in the substrate binding as well (underlined residues in Fig. 6A). A difference in this motif, worth investigating, is that in the thymidine kinases it is a conserved-ARY- triplet while in the deoxycytidine kinases it is a conserved -DRY- motif. One major difference in the substrate specificity site among these sequences is the distance and variation of the amino acids residues between the two

A



B

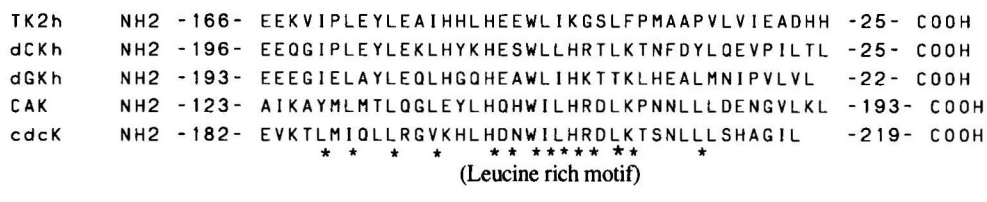


Fig. 6 A. Alignment of the amino acid sequences of six conserved sites in vertebrate herpes virus TKs, and comparison with human deoxyribonucleoside kinases. The sequences were aligned using DNA STAR program.
Abbreviations: HSV-1, herpes simplex virus type 1 (J02224); HSV-2, herpes simplex virus type 2 (J02225); ZVZ, varicella-zoster virus (M36160); EBV, Epstein-Barr virus (P03177); CCV, channel catfish virus (M75136); TK2h,human TK2; dCKh, human dCK; dGKh, human dGK.
B. Alignment of the sequences of the conserved leucine rich motif of human deoxynucleoside kinases with protein kinases; CAK, human CDK-activating kinase (A54820); cdcK, human cdc kinase (D54024). GeneBank accession numbers are given in the brackets for proper reference.

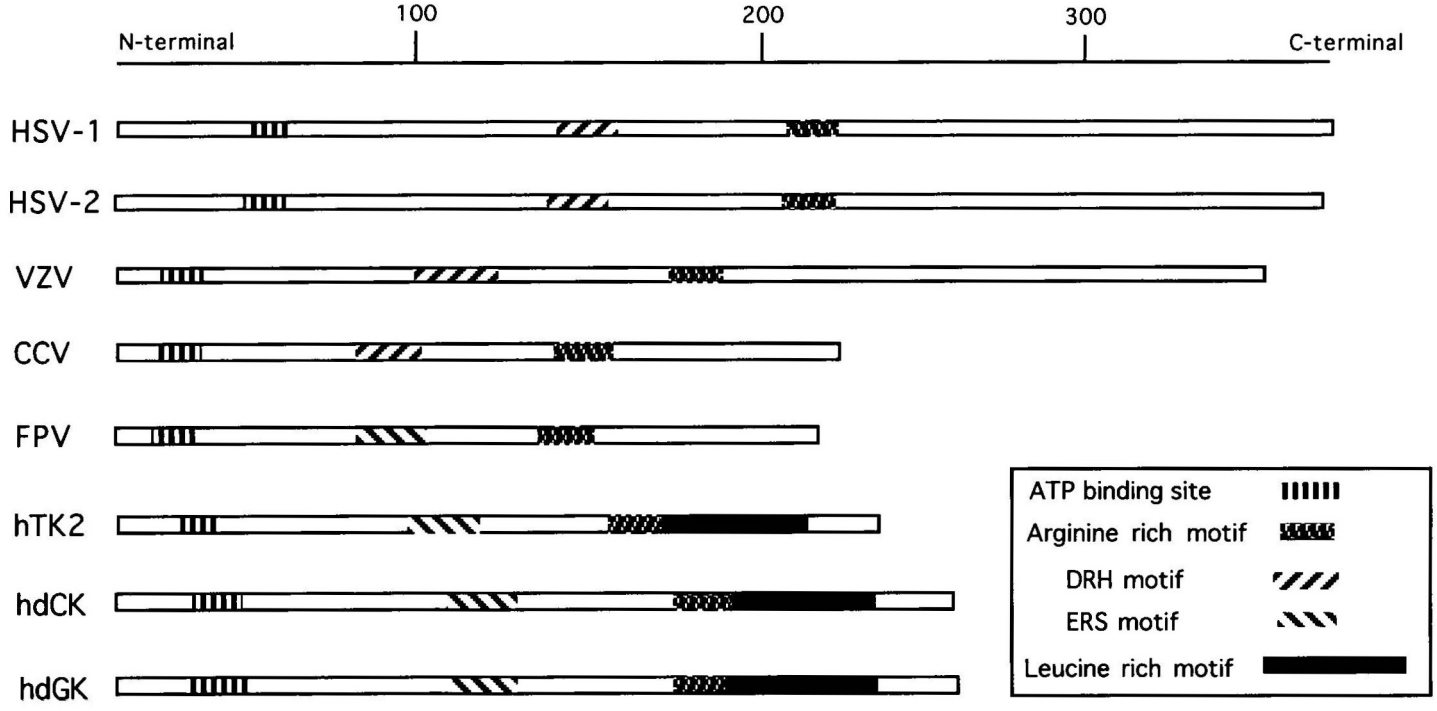


Fig. 7. Schematic overview of the arrangement of conserved sequence motifs along the entire sequences shown in Fig. 6.

conserved triplet sequence. The mammalian enzymes have much shorter distances, 3 amino acids compared to 10 amino acids in case of herpesviral TKs. This might be a reason why the mammalian enzymes fail to phosphorylate certain analogs such as acyclovir and ganciclovir. Future site-directed mutagenesis studies of the amino acid residues in these sites will help to define their roles in substrate recognition.

The arginine rich motif (site 5), similar to the ATP binding site, is conserved among all the sequence aligned in Fig. 6 and numerous ATP/GTP binding proteins. The conserved arginine residue is involved in the binding of the phosphoryl group as it does in adenylate kinase and HSV1 TK. The distance between the substrate specificity site and the arginine rich motif of the mammalian enzymes is only marginally longer than that of the herpes enzymes which suggests that they probably have the same function.

Site 2 is conserved among the human enzymes as well, suggesting that the conserved glutamic acid and tryptophan are involved in substrate binding like they are in HSV1 TK. Site 6 have not been assigned any function and it could only be found in TK2 but not in dCK and dGK, suggesting that TK2 is more closely related to the herpesvrial TKs.

The cytosolic thymidine kinase family

The properties of poxviral TKs and bacterial TKs are similar to the cytosolic TKs of vertebrates, so are their amino acids sequences (Gentry,1992). The human, chinese hamster, mouse and chicken TK1 sequence showed great similarities and conservation, only a few substitutions is seen. Poxviral TKs and eukaryotic cytosolic TKs are very similar, except that poxviral TKs are shorter both at the N-terminal and the C-terminal part. The bacterial TKs seemed to differ from their host TKs to some extend but still conserved sequence motif could be identified. The highly conserved ATP binding domain with the sequence finger print G/A-X-M-X-S/A-G-K-S/T close to the N-terminal was found in of all the sequences, as well as an arginine rich motif. However, the other substrate binding motifs indicated in Fig. 6 could not be identified. Detailed knowledge of the structure function relationships in the TK1 family must await 3-D structure determinations.

Origin of the mitochondrial salvage enzymes — TK2 and dGK

The mitochondrial location of TK2 and dGK

The subcellular localisation of TK2 and dGK is in the mitochondria as determined by enzyme activity measurement of subcellular fractions (Clayton, 1973; Gower *et al.*, 1979). However, both TK2 and dGK are encoded by nuclear genes. Like most mitochondrial proteins, they are synthesised in the cytoplasma as larger precursor proteins containing N-terminal leader peptides which will be removed by proteolytic cleavage upon entering the

mitochondria. Mitochondria have two membranes (inner and outer membranes) and therefor two compartments, *i. e.* the matrix and intermembrane spaces. Any imported precursor must be directed not only to the mitochondria but also to the correct intramitochondrial location. In a gene fusion experiments of van Loon *et al.* (1986) , it was demonstrated that the leader sequence of alcohol dehydrogenase III (ADHIII, a mitochondrial matrix enzyme) can direct mouse dihydrofolate reductase (DHFR, a cytosolic enzyme) into the mitochondrial matrix while the presequence of cytochrome c1 (a mitochondrial inner membrane protein protruding into the inner membrane space) transported the attached DHFR into the inner membrane space. There is no obvious amino acid homology between the signal peptide sequences, although they are typical rich in basic and hydroxylated amino acids and lack acidic residues (von Heijne *et al.*, 1989). Analysis of the presequence of dGK and presumed presequence of TK2 (since the N-terminal sequence of TK2 has not been defined yet) (Fig. 8) showed that they are

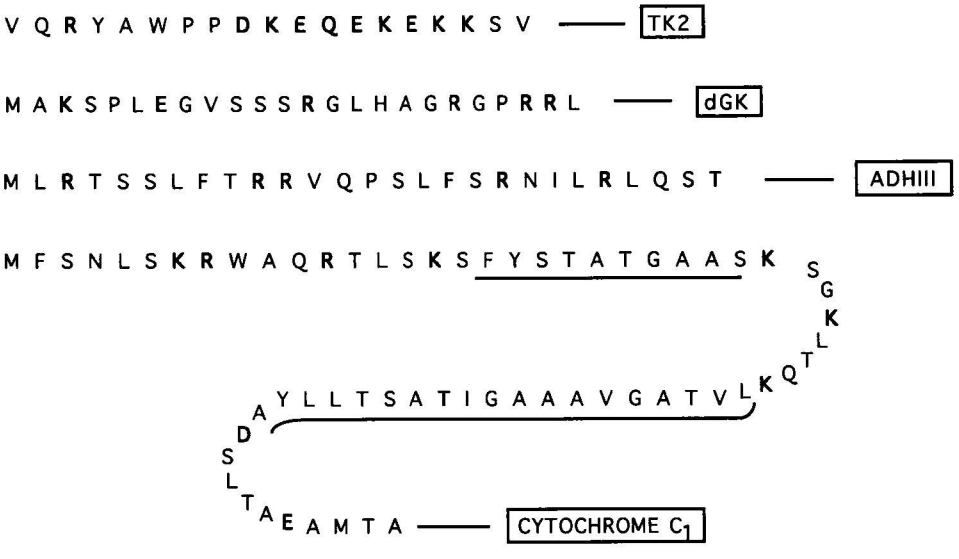


Fig. 8. Comparison of the N-terminal leader sequences of human TK2 and dGK to that of ADHIII and cytochrome c1. Charged amino acid residues are in bold letters. Two stretches of uncharged amino acid residues are underlined.

similar to the ADHIII presequence. They lack the stretch of uncharged amino acids typical for the cytochrome c1 presequence which is the signal for stop of transfer of intramembrane mitochondrial proteins. The location of both TK2 and dGK is most likely in the mitochondrial matrix.

The Origin of TK2 and dGK

According to the endosymbiotic theory, mitochondria once were free living bacteria like organism, similar to present day α purple bacteria, and they contained all the information necessary for growth and reproduction

(Margulis, 1970). Presumably all the proteins necessary to maintain the functional mitochondria were originally encoded in the mitochondrial genome. During evolution most, but not all, essential genetic information of the mitochondrial ancestor has been transferred to the nuclear genome, while part of the information was retained and still functioning today. Mammalian cells contain both TK2 and dGK located in the mitochondria. From the endosymbiotic point of view, the genes of TK2 and dGK were probably originally part of the mitochondria genome and subsequently transferred to nuclear genome of eukaryotic cells and lost from the mitochondria genome during evolution.

Many eukaryotic nuclear genomes have been reported to contain mitochondrial DNA like sequences, including locust (Gellissen *et al.*, 1983), yeast (Farrelly and Butow, 1983), podispora (Wright and Cummings, 1983), sea urchin (Jocobs *et al.*, 1983), maize (Kemble *et al.*, 1983), rat (Hadler *et al.*, 1983) and human (Tsuzuki *et al.*, 1983). It has been found that mtDNA of Neurospora contains DNA sequence homologous to the nuclear gene coding for one of the mitochondrial ATPase subunits (van den Boogaart *et al.*, 1982). In HeLa cells an intact replicative mitochondrial DNA was found in the nucleus (Keistensen and Prydz, 1986). In the rat nuclear genome a mitochondrial D-loop sequence was integrated (Zullo *et al.*, 1991). All these findings suggest that in eukaryotic cells physical contact between the two genomes, or between parts or copies of them, must have occurred during evolution. It was estimated that there are at least several hundred copies of mtDNA like regions per haploid human genome (Fukuda *et al.*, 1985).

It has been speculated that the integration of mtDNA fragments into nuclear DNA is mediated by mechanisms similar to those of viral DNA integration into host genomes. Analysis of the junctions of the mtDNA like sequences revealed that these mtDNA fragments did not integrate into specific sites on the nuclear genome (Nomiyama *et al.*, 1985; Fukuda *et al.*, 1985). However, the presence of highly repetitive sequences in the nuclear sequences flanking the mitochondrial homologous regions suggests a role in sequence mobility (Gellissen *et al.*, 1983; Wakasugi *et al.*, 1985; Nomiyama *et al.*, 1984). The eukaryotic genome is composed of up to 40% repetitive sequences (Britten and Kohne, 1968). It seems reasonable that mtDNA like sequences would be associated with repetitive sequences, as insertion into single-copy genes would probably be fatal to the gene and possible the organism, and repetitive sequences are favourable sites for integration of exongenous DNA (Kato *et al.*, 1986). It is likely that the TK2 gene was transferred from "pre-mitochondrion" to the nucleus at an early time since there is evidence for repetitive sequences in both the exon and intron sequences of TK2 (Wang *et al.*, unpublished data). These repetitive sequences may be movable elements, *i.e.* transposon-like, that are involved in the insertion of the gene into the nuclear DNA.

Such a gene translocation requires the addition of a mitochondrial target sequence to the open reading frame of the gene product, the correct positioning of cytoplasmic regulatory signals and the elimination of regulatory features specific to the organelle. The translocation of ribosomal protein S12 of *Oenothera* was studied and the transit sequence necessary to target this protein to the mitochondria is encoded by a 5'-extension of the open reading frame, separated by an intron from the corresponding mtDNA. Comparison of the amino acid sequence encoded by the nuclear gene with that encoded by the edited mitochondrial cDNA suggests that gene transfer between mitochondrion and nucleus started from an edited mitochondrial RNA molecules (Grohmann *et al.*, 1992). The mechanism for the gene transfer between mitochondria and the nucleus has been proposed for higher plants (Fig.9) (Brennicke *et al.*, 1993), and it is most likely also applicable to animal cells. There is an intron sequence that separate the functional mature protein from the presequence of dGK and at least 3 introns separate

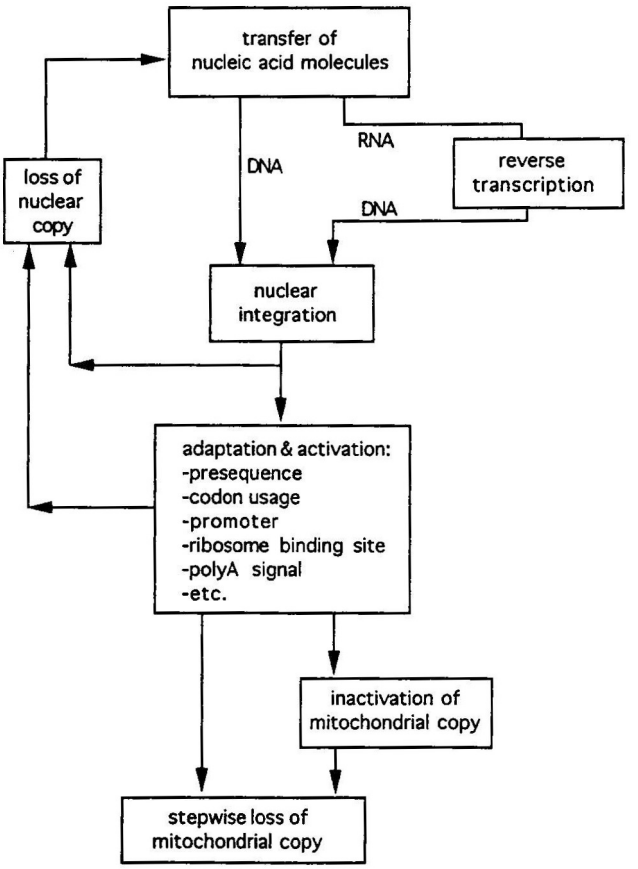


Fig. 9. The transfer of genetic information from mitochondrion to nucleus can be subdivided into different steps. Only complete passage through the entire procedure will result in a successful transfer, with the former organelle gene now actively integrated in the nucleus (Brennicke *et al.*, 1993).

the presequence of TK2 from the functional enzyme (Wang *et al.*, unpublished data). The existence of a repeated sequence in TK2 cDNA would allow us to speculate that the transfer of the TK2 gene from the "pre-mitochondria" genome to the nucleus followed a mechanism of RNA-mediated transfer. After integrating into the nuclear DNA presequences were added so that the protein can be directed to the mitochondrial location. More sequence information from other species than mammals, such as α purple bacteria, is needed to support the endosymbiotic origin of the mitochondrial deoxyribonucleoside kinases.

References

Anderson, M.M., Chen, J., Cole, C.N. & Conrad, S.E. 1996. Activation of the human thymidine kinase (TK) promoter by simian virus 40 large T antigen. *J. Virol. 70*, 6304-13.

Andres, C.M., Palella, T.D. & Fox, I.H. 1979. Human placental adenosine kinase: purification and characterization. *Adv. Exp. Med. Biol. 122B*, 41-3.

Arch, J.R. & Newsholme, E.A. 1978. Activities and some properties of 5'-nucleotidase, adenosine kinase and adenosine deaminase in tissues from vertebrates and invertebrates in relation to the control of the concentration and the physiological role of adenosine. *Biochem. J. 174*, 965-77.

Arnér, E.S., Spasokoukotskaja, T. & Eriksson, S. 1992. Selective Assay for Thymidine Kinase 1 and 2 and deoxycytidine Kinase and Their Activity in extracts from Human cells and tissues. *Biochem. Biophy. Res. Commun. 188*, 712-718.

Arnér, E.S.J. & Eriksson, S. 1995. Mammalian deoxyribonucleoside kinases. *Pharmac. Ther. 67*, 155-186.

Balasubramaniam, N.K., Veerisetty, V. & Gentry, G.A. 1990. Herpesviral deoxythymidine kinases contain a site analogous to the phosphoryl-binding arginine-rich region of porcine adenylate kinase; comparison of secondary structure predictions and conservation. *J. Gen. Virol. 71*, 2979-2987.

Bennett, L.L. & Hill, D.L. 1975. Structural requirements for activity of nucleosides as substrates for adenosine kinase: orientation of substituents on the pentofuranosyl ring. *Mol. Pharmacol. 11*, 803-8.

Berk, A.J. & Clayton, D.A. 1973. A Genetically Distinct Thymidine Kinase in Mammalian Mitochondria. *J. Biol. Chem. 248*, 2722-2729.

Bestwick, R.K., Moffet, G.L. & mathews, C.K. 1982. Selective expansion of mitochondrial nucleoside triphosphates pools in antimetabolite-treated Hela cells. *J. Biol. Chem 257*, 9300-4.

Björklund, S., Skog, S., Tribukait, B. & Thelander, L. 1990. S-phase-specific expression of mammalian ribonucleotide reductase R1 and R2 subunit mRNAs. *Biochemistry 29*, 5452-8.

Black, M.E., Newcomb, T.G., Wilson, H.-M.P. & Loeb, L.A. 1996. Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy. *Proc. Natl. Acad. Sci. USA 93*, 3525-3529.

Black, M.E., Rechtin, T.M. & Drake, R.R. 1996. Effect on substrate binding of an alteration at the conserved aspartic acid-162 in herpes simplex virus type 1 thymidine kinase. *J. Gen. Virol. 77*, 1521-1527.

Bogenhagen, D. & Clayton, D.A. 1976. Thymidylate nucleotide supply for mitochondrial DNA synthesis in mouse L-cells. Effect of 5-fluorodeoxyuridine and methotrexate in thymidine kinase plus and thymidine kinase minus cells. *J. Biol. Chem. 251*, 2938-44.

Bohman, C. & Eriksson, S. 1988. Deoxycytidine Kinase from Human leukemic Spleen: Preparation and characterization of the Homogeneous Enzyme. *Biochemistry 27*, 4258-4265.

Bohman, C. & Eriksson, S. 1990. Mammalian deoxynucleoside kinases. *Biochem. (Life Sci. Adv.) 9*, 11-35.

Bontemps, F., Mimouni, M. & Berghe, G. Van den. 1993. Phosphorylation of adenosine in anoxic hepatocytes by an exchange reaction catalysed by adenosine kinase. *Biochem. J. 290*, 679-84.

Boogaart, P.van den, Samallo, J. & Agsteribbe, E. 1982. Similar genes for a mitochondrial ATPase subunit in the nuclear and mitochondrial genomes of Neurospora crassa. *Nature 298*, 187-189.

Bradshaw, H.D. 1983. Molecular cloning and cell cycle-specific regulation of a functional human thymidine kinase gene. *Proc. Natl. Acad. Sci. U S A 80*, 5588-91.

Bradshaw, H.D. & Deininger, P.L. 1984. Human thymidine kinase gene: molecular cloning and nucleotide sequence of a cDNA expressible in mammalian cells. *Mol. Cell. Biol. 4*, 2316-20.

Brennicke, A., Grohmann, L., Hiesel, R., Knoop, V. & Schuster, W. 1993. The mitochondrial genome on its way to the nucleus: different stages of gene transfer in higher plants. *FEBS lett. 325*, 140-145.

Britten, R.J. & Kohne, D.E. 1968. Repeated sequences in DNA. *Science 161*, 529-540.

Brown, D.G., Visse, R., Sandhu, G., Davies, A., Rizkallah, P.J., Melitz, C., Summers, W.C. & Sanderson, M.R. 1995. Crystal structures of the thymidine kinase from herpes simplex virus type-1 in complex with deoxythymidine and ganciclovir. *Nat. Struct. Biol. 2*, 876-881.

Caldwell, I.C., Henderson, J.F. & Paterson, A.R.P. 1967. *Can. J. Biochem. 45*, 735-744.

Carson, D. A., Wasson, D. B., Kaye, J., Ullman, B., Martin, D. W., Robins, R. K. & Montgomery, J. A. (1980). Deoxycytidine kinase-mediated toxicity of deoxyadenosine analogs toward malignant human lymphoblasts in vitro and toward murine L1210 leukemia in vivo. *Proc. Natl. Acad. Sci. USA* , 6865-9.

Chan, T., Ishii, K., Long, C. & Green, H. 1973. Purine excretion by mammalian cells deficient in adenosine kinase. *J. Cell Physiol. 81*, 315-321.

Chang, L.S.M. & Bollum, F.J. 1972. Variation of deoxyribonucleic acid polymerase activities during rat liver regeneration. *J. Biol. Chem. 247*,

Chang, Z.-F. & Huang, D.-Y. 1996. The regulation of thymidine kinase in HL-60 human promyeoleukemia cells. *J. Biol. Chem. 268*, 1266-1271.

Chang, Z.F. 1990. Post-transcriptional regulation of thymidine kinase gene expression during monocytic differentiation of HL60 promyelocytes. *Biochem. Biophys. Res. Commun. 169*, 780-7.

Chang, Z.F. & Cheng, S.M. 1993. Constitutive overexpression of DNA binding activity to the distal CCAAT box of human thymidine kinase promoter in human tumor cell lines. *Cancer Res. 53*, 3253-6.

Chang, Z.F. & Huang, D.Y. 1993. The regulation of thymidine kinase in HL-60 human promyeloleukemia cells. *J. Biol. Chem. 268*, 1266-71.

Chang, Z.F., Huang, D.Y. & Hsue, N.C. 1994. Differential phosphorylation of human thymidine kinase in proliferating and M phase-arrested human cells. *J. Biol. Chem. 269*, 21249-21254.

Chen, E.H., JohnsonII, E.E., Vetter, S.M. & Mitchell, B.S. 1995. Characterization of the deoxycytidine kinase promoter in human lymohoblast cell lines. *J. Clin. Invest. 95*, 1660-1668

Chen, S.H., Shine, H.D., Goodman, J.C., Grossman, R.G. & Woo, S.L. 1994. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer in vivo. *Proc Natl Acad Sci USA 91*, 3054-7.

Cheng, Y.C., Domin, B. & Lee, L.S. 1977. Human deoxycytidine kinase. Purification and characterization of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia patients. *Biochim Biophys Acta 481*, 481-92.

Chiu, R.W. & Baril, E.F. 1975. Nuclear DNA polymerases and HeLa cell cycle. *J. Biol. Chem. 250*, 7951-7957.

Chottiner, E.G., Shewach, D.S., Datta, N.S., Ashcraft, E., Gribbin, D., Ginsburg, D., Fox, I.H. & Mitchell, B.S. 1991. Cloning and expression of human deoxycytidine kinase cDNA. *Proc. Natl. Acad. Sci. USA 88*, 1531-1535.

Cohen, H., Hirschhorn, R., Horowitz, S., Rubinstein, A., Polmar, S.H., Hong, R. & Jr., D.W.M. 1978. *Proc. Natl. Acad. Sci. USA 75*, 472-476.

Datta, A.K., Bhaumik, D. & Chatterjee, R. 1987. Isolation and characterization of adenosine kinase from Leishmania donovani. *J Biol Chem 262*, 5515-21.

Datta, N.S., Shewach, D.S., Mitchell, B.S. & Fox, I.H. 1989. Kinetic properties and inhibition of human T lymphoblast deoxycytidine kinase. *J. Biol. Chem. 264*, 9359-9364.

Elledge, S.J., Zhou, Z. & Allen, J.B. 1992. Ribonucleitide reductase: Regulation, regulation, regulation. *TIBS 17*, 119-123.

Engström, Y., Eriksson, S., Jildevik, I., Skog, S., Thelander, L. & Tribukait, B. 1985. Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits. *J Biol Chem 260*, 9114-6.

Eriksson, S., Gräslund, A., Skog, S., Thelander, L. & Tribukait, B. 1984. Cell cycle-dependent regulation of mammalian ribonucleotide reductase. The S phase-correlated increase in subunit M2 is regulated by de novo protein synthesis. *J Biol Chem 259*, 11695-700.

Eriksson, S., Kierdaszuk, B., Munch-Petersen, B., Öberg, B. & Johansson, N.G. 1991. Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. *Biochem Biophys Res Commun 176*, 586-92.

Eriksson, S., Wang, J., Gronowitz, S. & Johansson, N.G. 1995. Substrate specificities of mitochondrial thymidine kinase and cytosolic deoxycytidine kinase against 5-aryl substituted pyrimidine 2'-deoxyribose analogues. *Nuclsides & Nucleotides 14*, 507-10.

Ezzeddine, Z.D., Martuza, R.L., Platika, D., Short, M.P., Malick, A., Choi, B. & Breakefield, O. 1991. Selective killing of glioma cells in culture and in vivo by retrovirus transfer of the herpes simplex virus thymidine kinase gene. *New Biol 3*, 608-14.

Farrelly, F. & Butow, R.A. 1983. Rearranged mitochondrial genes in the yeast nuclear genome. *Nature 301*, 296-301.

Fisher, M.N. & Newsholme, E.A. 1984. Properties of rat heart adenosine kinase. *Biochem J 221*, 521-8.

Flasshove, M., Strumberg, D., Ayscue, L., Mitchell, B.S., C. Tirier, W.H., Seeber, S. & Schutte, J. 1994. Structural analysis of the deoxycytidine kinase gene in patients with acute myeloid leukemia and resistance to cytosine arabinoside. *Leukemia 8*, 780-5.

Flemington, E., Bradshaw, H.D., Traina-Dorge, V., Slagel, V. & Deininger, P.L. 1987. Sequence, structure and promoter characterization of the human thymidine kinase gene. *Gene 52*, 267-77.

Fukuda, M., Wakasugi, S., Tsuzuki, T., Nomiyama, H. & Shimada, K. 1985. Mitochondrial DNA-like sequences in the human nuclear genome Characterization and implications in the evolution of mitochondrial DNA. *J. Mol. Biol. 186*, 257-266.

Furman, P.A., Fyfe, J.A., Clair, M.H.S., Weinhold, K., Rideout, J.L., Freeman, G.A., Lehrma, S.N., Bolognesi, D.P., Broder, S., Mitsuya, H. & Barry, D.W. 1986. Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA 83*, 8333-7.

Gellissen, G., Bradfield, J.Y., White, B.N. & Wyatt, G.R. 1983. Mitochondrial DNA sequences in the nuclear genome of a locust. *Nature 301*, 631-634.

Gentry, G.A. 1992. Viral thymidine kinases and their relatives. *Pharmac. ther. 54*, 319-355.

Gower, W.J., Carr, M.C. & Ives, D.H. 1979. Deoxyguanosine kinase. Distinc molecular forms in mitochondria and cytosol. *J. Biol. Chem. 154*, 2180-2183.

Greenberg, G.R. & Hilfinger, J.M. 1996. Regulation of synthesis of ribonucleotide reductase and relationship to DNA replication in various systems. *Prog. Nucleic Acid Res. Mol. Biol. 53*, 345-395.

Grohmann, L., Brennicke, A. & Schuster, W. 1992. The mitochindrial gene encoding ribosomal protein S12 has been translocated to the nuclear genome in *Oenothera*. *Nucleic Acids Res. 20*, 5641-5646.

Gudas, J.M., Fridovich-Keil, J.L. & Pardee, A.B. 1993. Posttranscriptional control of thymidine kinase messenger RNA accumulation in cells released from G0-G1 phase blocks. *Cell Growth Differ 4*, 421-30.

Hadler, H.I., Dimitrijevic, B. & Mahalingam, R. 1983. *Proc. Natl. Acad. Sci. USA 80*, 6495-6499.

Hapke, D.M., Stegmann, A.P.A. & Mitchell, B.S. 1996. Retroviral transfer of deoxycytidine kinase into tumor cell lines enhancec nucleoside toxicity. *Cancer Res. 56*, 2343-2347.

Harrison, P.T., Thompson, R. & Davison, A.J. 1991. Evolution of herpesvirus thymidine kinases from cellular deoxycytidine kinase. *J. Gen. Virol. 72*, 2583-2586.

Hawkins, C.F. & Bagnara, A.S. 1987. Adenosine kinase from human erythrocytes: kinetic studies and characterization of adenosine binding sites. *Biochemistry 26*, 1982-7.

Hebert, M.E., Greenberg, M.L., Chaffee, S., Gravatt, L., Hershfield, M.S., Elion, G.B. & Kurtzberg, J. 1991. Pharmacologic purging of malignant T cells from human bone marrow using 9-beta-D-arabinofuranosylguanine. *Transplantation 52*, 634-40.

Heijne, G.von, Steppuhn, J. & Herrmann, R.G. 1989. Domain structure of mitochondrial and chloroplast targeting peptides. *Eur. J. Biochem. 180*, 535-545.

Heintz, N.H. & Stillman, B.W. 1988. Nuclear DNA synthesis in vitro is mediated via stable replication forks assembled in a temporally specific fashion in vivo. *Mol Cell Biol 8*, 1923-31.

Hengstschläger, M., Denk, C. & Wawra, E. 1993. Cell cycle regulation of deoxycytidine kinase-evidence for post-transcriptional control. *FEBS lett 321*, 237-240.

Hengstschläger, M., Knofler, M., Mullner, E.W., Ogris, E., Wintersberger, E. & Wawra, E. 1994. Different regulation of thymidine kinase during the cell cycle of normal versus DNA tumor virus-transformed cells. *J Biol Chem 269*, 13836-42.

Hengstschläger, M., Mudrak, I., Wintersberger, E. & Wawra, E. 1994. A common regulation of genes encoding enzymes of the deoxynucleotide metabolism is lost after neoplastic transformation. *Cell Growth & Differ. 5*, 1389-1394.

Hengstschläger, M., Pusch, O., Hengstschläger-Ottnad, E., Ambros, P.F., Bernaschek, G. & Wawra, E. 1996. Loss of the p16/MTS1 tumor suppressor gene causes E2F-mediated deregulation of essential enzymes of the DNA precursor metabolism. *DNA Cell Biol. 15*, 41-51.

Hong, Y.S., Ma, G.T. & Ives, D.H. 1995. Directed mutagenesis of deoxyguanosine site at arginine 79 up-regulated turnover on deoxyadenosine kinase subunit of heterodimeric enzyme from *Lactobacillus acidophilus* R26. *J. Biol. Chem. 270*, 6602-6606.

Jensen, H.K. & Munch-Petersen, B. 1994. Altered kinetic properties of recombinant human cytosolic thymidine kinase (TK1) as compared with the native form. *Adv Exp Med Biol 370*, 637-640.

Jocobs, H.T., Posakony, J.W., Grula, J.W., Roberts, J.W., Xin, J.-H., Britten, R.J. & Davidson, E.H. 1983. Mitochondrial DNA sequences in the nuclear genome of Strongylocentrotus purpuratus. *J. Mol. Biol. 165*, 609-632.

Johansson, M. & Karlsson, A. 1996. Cloning and expression of human deoxyguanosine kinase cDNA. *Proc. Natl. Acad. Sci. USA 93*, 7258-7262.

Johnson, W.A. & Fridland, A. 1989. Phosphorylation of 2',3'-Dideoxyinosine by Cytosolic 5'-Nucleotidase of Humam Lymphoid Cells. *Molecular Pharmacology 36*, 291-295.

Karlsson, A., Johansson, M. & Eriksson, S. 1994. Cloning and expression of mouse deoxycytidine kinase. *J. Biol. Chem. 269*, 24374-24378.

Kato, S., Anderson, R.A. & Camerini-otero, R.D. 1986. Foreign DNA introduced by calcium phosphate is intefrated into repetitive DNA elements of the mouse L cell genome. *Mol. Cell. Biol. 6*, 1787-1795.

Kauffman, M.G. & Kelly, T.J. 1991. Cell cycle regulation of thymidine kinase: residues near the carboxyl terminus are essential for the specific degradation of the enzyme at mitosis. *Mol Cell Biol 11*, 2538-46.

Kauffman, M.G., Rose, P.A. & Kelly, T.J. 1991. Mutations in the thymidine kinase gene that allow expression of the enzyme in quiescent (G0) cells. *Oncogene 6*, 1427-35.

Keistensen, T. & Prydz, H. 1986. The presence of intact mitochondrial DNA in HeLa cell Nuclei. *Nucleic Acids Res. 14*, 2597-609.

Kemble, R.J., Mans, R.J., Gabay-Laughnan, S. & Laughnan, J.R. 1983. *Nature 304*, 744-747.

Kim, M.-Y. & Ives, D.H. 1989. Human deoxycytidine kinase: kinetic mechanism and end product regulation. *Biochemistry 28*, 9043-9047.

Kitos, T.E. & Tyrrell, D.L.J. 1995. Intracellular metabolism of 2',3'-dideoxynucleosides in duck hepatocyte primary cultures. *Biochem. Pharmacol. 49*, 1291-1302.

Kraweic, K., Kierdaszuk, B., Eriksson, S., Munch-Petersen, B. & Shugar, D. 1995. Nucleoside triphosphate donors for nucleoside kinases: Donor properties of UTP with human deoxycytidine kinase. *Biochem. Biophys. Res. Commun 216*, 42-48.

Kreidberg, J.A. & Kelly, T.J. 1986. Genetic analysis of the human thymidine kinase gene promoter. *Mol Cell Biol 6*, 2903-9.

Kunz, B.A. 1988. Mutagenesis and deoxyribonucleotide pool imbalance. *Mutat Res 200*, 133-47.

Kurtzberg, J. 1993. Guanine arabinoside as a bone marrow-purging agent. *Ann N Y Acad Sci 685*, 225-36.

Kurtzberg, J., Wiley, J., Reese, M., Elion, G. & Mitchell, B.S. 1995. *Blood 86*, Suppl. 1, 511a.

Lambe, C.U., Averett, D.R., Paff, M.T., Reardon, J.E., Wilson, J.G. & Krenitsky, T.A. 1995. 2-Amino-6-methoxypurine arabinoside: an agent for T-cell malignancies. *Cancer Res 55*, 3352-3356.

Lecrenier, N. & Foury, F. 1995. Overexpression of the RNR1 gene rescues Saccharomyces cerevisiae mutants in the mitochondrial DNA polymerase-encoding MIP1 gene. *Mol Gen Genet 249*, 1-7.

Loon, A.P.G.M.van, Brändii, A.W. & Schatz, G. 1986. The presequences of two imported mitochondrial proteins contain information for intracellular and intramitochondrial sorting. *Cell 44*, 802-812.

Ma, G.T., Hong, Y.S. & Ives, D.H. 1995. Cloning and expression of the heterodimeric deoxyguanosine kinase/deoxyadenosine kinase of *Lactobacillus acidophilus* R-26. *J. Biol. Chem. 270*, 6595-6601.

Manome, Y., Wen, P.Y., Dong, Y., Tanaka, T., Mitchell, B.S., Kufe, D.W. & Fine, H.A. 1996. Viral vector transduction of the human deoxycytidine kinase cDNA sensitizes glioma cells to the cytotoxic effects of cytosine arabinoside *in vitro* and *in vivo*. *Nat. Med. 2*, 567-573.

Margulis, L.1970 *Origins of Eukaryotic Cells.* New Haven: Yale University Press.

Matthes, E., Lehmann, C., Scholz, D., Rosenthal, H.A. & Langen, P. 1988. Phosphorylation, anti-HIV activity and cytotoxicity of 3'-fluorothymidine. *Biochem Biophys Res Commun 153*, 825-31.

McDougall, J.K., Kucherlapati, R. & Ruddle, F.H. 1973. Localization and induction of the human thymidine kinase gene by adenovirus 12. *Nature New Biol 245*, 172-5.

Meuth, M. 1984. The genetic consequences of nucleotide precursor pool imbalance inmammalian cells. *Mutat Res 126*, 107-12.

Meuth, M. 1989. The molecular basis of mutations induced by deoxyribonucleoside triphosphate pool imbalances in mammalian cells. *Exp Cell Res 181*, 305-16.

Mikulits, W., Hengstschläger, M., Sauer, T., Wintersberger, E. & Mullner, E.W. 1996. Overexpression of thymidine kinase mRNA eliminates cell cycle regulation of thymidine kinase enzyme activity. *J. Biol. Chem. 271*, 853-60.

Mikulits, W. & Mullner, E.W. 1994. Post-transcriptional repression of thymidine kinase expression during cell cycle and growth stimulation. *FEBS Lett 345*, 172-6.

Miller, R.L., Adamczyk, D.L. & Miller, W.H. 1979. Adenosine kinase from rabbit liver. I. Purification by affinity chromatography and properties. *J Biol Chem 254*, 2339-45.

Miller, R.L., Adamczyk, D.L., Miller, W.H., Koszalka, G.W., Rideout, J.L., Beacham, L.M., Chao, E.Y., Haggerty, J.J., Krenitsky, T.A. & Elion, G.B. 1979. Adenosine kinase from rabbit liver. II. Substrate and inhibitor specificity. *J Biol Chem 254*, 2346-52.

Mimouni, M., Bontemps, F. & Van den Berghe, G. 1994. Kinetic studies of rat liver adenosine kinase. Explanation of exchange reaction between adenosine and AMP. *J. Biol. Chem 269*, 17820-5.

Mitchell, B.S., Song, J.J., Johnson-II, E.E., Chen, E. & Dayton, J.S. 1993. Regulation of human deoxycytidine kinase expression. *Advan. Enzyme Regul. 33*, 61-68.

Munch-Petersen, B., Cloos, L., Tyrsted, G. & Eriksson, S. 1991. Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. *J. Biol. Chem. 266*, 9032-9038.

Munch-Petersen, B., L. Cloos, H.K.J. & Tyrsted, G. 1995. Human thymidine kinase 1. Regulation in normal and malignant cells. *Adv. Enzyme Regul. 35*, 69-89.

Munch-Petersen, B., Tyrsted, G. & Cloos, L. 1993. Reversible ATP-dependent transition between two forms of human cytosolic thymidine kinase with different enzymatic properties. *J. Biol. Chem. 268*, 15621-5.

Munch-Petersen, B., Tyrsted, G., Cloos, L., Beck, R.A. & Eger, K. 1995. Different affinity of the two forms of human cytosolic thymidine kinase towards pyrimidine analogs. *Biochim Biophys Acta 1250*, 158-162.

Nishizawa, T., Nishida, Y. & Akaoka, I. 1976. *Clin. Chim. Acta 67*, 15-20.

Nomiyama, H., Fukuda, M., Wakasugi, S., Tsuzuki, T. & Shimada, K. 1985. Molecular structure of mitochondrial-DNA-like sequences in human nuclear DNA. *Nucleic Acids Res. 13*, 1649-1658.

Nomiyama, H., Tsuzuki, T., Wakasugi, S., Fukuda, M. & Shimada, K. 1984. Interruption of a human nuclear sequence homologous to mitochondrial DNA by a member of the KpnI 1.8 kb family. *Nucleic Acids Res 12*, 5225-34.

Ogasawara, N., Yamada, Y. & Goto, H. 1979. Adenosine and deoxyadenosine kinase from rat liver. *Adv Exp Med Biol 122B*, 151-6.

Ogris, E., Rotheneder, H., Mudrak, I., Pichler, A. & Wintersberger, E. 1993. A binding site for transcription factor E2F is a target for trans activation of murine thymidine kinase by polyomavirus large T antigen and plays an important role in growth regulation of the gene. *J Virol 67*, 1765-71.

Oliver, F.J., Collins, M.K.L. & Lopez-rivas, A. 1996. Regulation of the salvage pathway of deoxynucleotides synthesis in apoptosis induced by growth factor deprivation. *Biochem. J. 316*, 421-425.

Osborne, W.R.A. & Scott, C.R. 1983. The metabolism of deoxyguanosine and guanosine in human B and T lymphoblasts. *Biochem. J. 214*, 711-718.

Owens, J.K., Shewach, D.S., Ullman, B. & Mitchell, B.S. 1992. Resistance to 1-β-D-arabinofuranosylcytosine in human T-lymphoblasts mediated by mutations within the deoxycytidine kinase gene. *Cancer Res. 52*, 2389-2393.

Palella, T.D., Andres, C.M. & Fox, I.H. 1980. Human placental adenosine kinase. Kinetic mechanism and inhibition. *J Biol Chem 255*, 5264-9.

Pang, J.H. & Chen, K.Y. 1993. A specific CCAAT-binding protein, CBP/tk, may be involved in the regulation of thymidine kinase gene expression in human IMR-90 diploid fibroblasts during senescence. *J Biol Chem 268*, 2909-16.

Park, I. & Ives, D.H. 1988. Properties of a Highly Purified Mitochondrial Deoxyguanosine Kinase. *Arch. Biochem. Biophys. 266*, 51-60.

Park, I. & Ives, D.H. 1995. Kinetic mechanism and end-product regulation of deoxyguanosine kinase from beef liver mitochondria. *J. Biol. chem. 117*, 1058-1061.

Plagemann, P.G. & Woffendin, C. 1989. Permeation and salvage of dideoxyadenosine in mammalian cells. *Mol. Pharmacol. 36*, 185-92.

Plagemann, P.G., Wohlhueter, R.M. & Woffendin, C. 1988. Nucleoside and nucleobase transport in animal cells. *Biochim. Biophys. Acta 947*, 405-443.

Reichard, P. 1988. Intractions between deoxyribonucleotide and DNA synthesis. *Annu. Rev. Biochem. 57*, 349-374.

Rotllan, P. & Portugal, M.T.M. 1985. Adenosine kinase from bovine adrenal medulla. *Eur. J. Biochem. 151*, 365-71.

Ruiz van Haperen, V.W.T., Veeman, G., Eriksson, S., Boven, E., Stegmann, A.P.A., Hermsen, M., Vermorken, J.B., Pinedo, H.M. & Peters, G.J. 1994. Development and molecular characterization of a 2', 2'-difluorodeoxycytidine resistant variant of the human ovarian carcinoma cell line A2780. *Cancer Res. 54*, 4138-4143.

Ruiz van Haperen, V.W.T., Veerman, G., Eriksson, S., Stegmann, A.P. & Peters, G.J. 1995. Induction of resistance to 2',2'-difluorodeoxycytidine in the human ovarian cancer cell line A2780. *Semin. Oncol. 22*, 35-41.

Ruiz van Haperen, V.W.T., Veerman, G., Vermorken, J.B., Pinedo, H.M. & Peters, G.J. 1996. Regulation of phosphorylation of deoxycytidine and 2', 2'-difluorodeoxycytidine (Gemcitabine); Effects of cytidine 5'-triphosphate and uridine triphosphate in relation to chemosensitivity for 2',2'-difluorodeoxycytidine. *Biochem. Pharmacol. 51*, 911-18.

Sarup, J.C. & Fridland, A. 1987. Identification of Purine deoxyribonucleoside Kinases from Human Leukemia Cells: Substrate Activation by Purine and Pyrimidine Deoxyribonucleosides. *Biochemistry 26*, 590-597.

Sayos, J., Solsona, C., Mallol, J., Lluis, C. & Franco, R. 1994. Phosphorylation of adenosine in renal brush-border membrane vesicles by an exchange reaction catalysed by adenosine kinase. *Biochem. J. 297*, 491-6.

Schnebli, H.P., Hill, D.L. & Bennett, L.L. 1967. Purification and properties of adenosine kinase from human tumor cells of type H. Ep. No. 2. *J. Biol. Chem. 242*, 1997-2004.

Shay, J.W., Pierce, D.J. & Werbin, H. 1990. Mitochondrial DNA copy number is proportional to total cell DNA under a variety of growth conditions. *J. Biol. Chem. 265*, 14802-7.

Shewach, D.S., Daddona, P.E., Ashcraft, E. & Mitchell, B.S. 1985. Metabolism and selective cytotoxicity of 9-beta-D-arabinofuranosylguanine in human lymphoblasts. *Cancer Res. 45*, 1008-14.

Shewach, D.S. & Mitchell, B.S. 1989. Differential Metabolism of 9-β-D-Arabinofuranosylguanine in Human Leukemic Cells. *Cancer Res. 49*, 6498-6502.

Shewach, D.S., Reynolds, K.K. & Hertel, L. 1992. Nucleotide specificity of human deoxycytidine kinase. *Mol. Pharmacol. 42*, 518-524.

Shioda, M. 1988. Subcellular localization of DNA polymerase γ and changes in its activity in sea urchin embryos. *Comp. Biochem. Physiol. 91B*, 525-530.

Snyder, F.F., Jenuth, J.P., Dilay, J.E., Fung, E., Lightfoot, T. & Mably, E.R. 1994. Secondary loss of deoxyguanosine kinase acticity in purine nucleoside phosphorylase deficient mice. *Biochim. Biophys. Acta 1227*, 33-40.

Snyder, F.F. & Lukey, T. 1982. Kinetic considerations for the regulation of adenosine and deoxyadenosine metabolism in mouse and human tissues based on a thymocyte model. *Biochim. Biophys. Acta 696*, 299-307.

Song, J.J., Walker, S., Chen, E., Johnson, E.E., Spychala, J., Gribbin, T. & Mitchell, B.S. 1993. Genomic structure and chromosomal localization of the human deoxycytidine kinase gene. *Proc. Natl. Acad Sci. USA 90*, 431-434.

Spychala, J., Datta, N.S., Takabayashi, K., Datta, M., Fox, I.H., Gribbin, T. & Mitchell, B.S. 1996. Cloning of human adenosine kinase cDNA: Sequence similarity to microbial ribokinases and fructokinases. *Proc. Natl. Acad. Sci. UAS 93*, 1232-1237.

Stegmann, A.P.A., Honders, M.W., Willemze, R. & Landegent, J.E. 1994. Cloning of the Dck gene encoding rat deoxycytidine kinase. *Gene 150*, 351-354.

Stegmann, A.P.A., Honders, M.W., Bolk, M.W.J., Wessels, J., Willemze, R. & Landegent, J.E. 1993. Assignment of the human deoxycytidine kinase (DCK) gene to chromosome 4 Band q13.3-q21.1. *Genomics 17*, 528-529.

Stegmann, A.P.A., Willemze, W.H., Ruiz van Haperen, V.W.T. & Landegent, J.E. 1995. Transfection of wild type deoxycytidine kinase (dck)cDNA into an araC-and DAC-resistant rat leukemic cell line of clonal origin fully restores drug sensitivity. *Blood 85*, 1188-94.

Stewart, C.J., Ito, M. & Conrad, S.E. 1987. Evidence for transcriptional and post-transcriptional control of the cellular thymidine kinase gene. *Mol. Cell Biol. 7*, 1156-63.

Stubbe, J. 1990. Ribonucleotide reductase: amazing and confusing. *J. Biol. Chem. 265*, 5329-5332.

Suzutani, T., Davies, L.C. & Honess, R.W. 1993. Kinetic studies of the predicated substrate-binding site of varicella-zoster virus thymidine kinase. *J. Gen. Virol. 74*, 1011-1016.

Terai, C., Wasson, D.B., Carrera, C.J. & Carson, D.A. 1991. Dependence of cell survival on DNA repair in human mononuclear phagocytes. *J. Immunol. 147*, 4302-4306.

Thelander, L. & Reichard, P. 1979. Reduction of ribonucleotides. *Annu. Rev. Biochem. 48*, 133-158.

Tsuzuki, T., Nomiyama, H., Setoyama, C., Maeda, S. & Shimada, K. 1983. Presence of mitochondrial-DNA-like sequences in the human nuclear DNA. *Gene 25*, 223-229.

Wakasugi, S., Nomiyama, H., Fukuda, M., Tsuzuki, T. & Shimada, K. 1985. Insertion of a long KpnI family member within a mitochondrial-DNA-like sequence present in the human nuclear genome. *Gene 36*, 281-8.

Wakazono, Y., Kubota, M., Furusho, K., Liu, L. & Gerson, S.L. 1996. Thymidine kinase deficient cells with decreased TTP pools are hypersensitive to DNA alkylating agents. *Mutat. Res. 362*, 119-125.

Wallace, D.C. 1992. Diseases of the mitochondrial DNA. *Annu Rev Biochem 61*, 1175-212.

Wang, J. & Eriksson, S. 1996. Phosphorylation of the anti-hepatitis B nucleoside analog 1-(2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil(FIAU) by human cytosolic and mitochondrial thymidine kinase and implication for cytotoxicity. *Antimicro. ag. Chemther. 40*, 1555-1557.

Wang, L., Hellman, U. & Eriksson, S. 1996. Cloning and expression of human mitochondrial deoxyguanosine kinase cDNA. *FEBS lett. 390*, 39-43.

Wang, L., Herrström Sjöberg, A., Bergman, T., Hellman, U. & Eriksson, S. 1996. Cloning and expression of human mitochondrial thymidine kinase cDNA. *(Manuscript)*

Wang, L.-M., Kucera, G.L. & Capizzi, R.L. 1993. Purification and characterization of deoxycytidine kinase from acute myeloid leukemia cell mitochondria. *Biochim. Biophy. Acta 1202*, 309-316.

Weissbach, A. 1977. Eukaryotic DNA polymerases. *A. Rev. Biochem. 46*, 25-47.

White, J.C. & Capizzi, R.L. 1991. A critical role for uridine nucleotide in the regulation of deoxycytidine kinase and the concentration dependence of 1-β-D-arabinofuranosylcytosine phosphorylation in human leukemia cells. *Cancer Res. 51*, 2559-2665.

Wild, K., Bohner, T., Aubry, A., Folkers, G. & Schulz, G.E. 1995. The three -dimensional structure of thymidine kinase from herpes simplex virus type 1. *FEBS lett. 368*, 289-292.

Willecke, K., Teber, T., Kucherlapati, R.S. & Ruddle, F.H. 1977. Human mitochondrial thymidine kinase is coded for by a gene on chromosome16 of the nucleus. *Somatic Cell Genet. 3*, 237-45.

Wills, K.N., Huang, W.M., Harris, M.P., Machemer, T., Maneval, D.C. & Gregory, R.J. 1995. Gene therapy for hepatocellular carcinoma: chemosensitivity conferred by adenovirus-mediated transfer of the HSV-1 thymidine kinase gene. *Cancer Gene Ther. 2*, 191-197.

Wintersberger, E., Rotheneder, H., Grabner, M., Beck, G. & Seiser, C. 1992. Regulation of thymidine kinase during growth, cell cycle and differentiation. *Adv. Enzyme Regul. 32*, 241-54.

Wright, R.M. & Cummings, D.J. 1983. Integration of mitochondrial gene sequences within the nuclear genome during senescence in a fungus. *Nature 302*, 86-88.

Xia, L., Liang, G., Liu, W.Z., Yuan, Y. & Lipson, K.E. 1995. Localization of a cycloheximide-sensitivity response element in the human thymidine kinase gene promoter. *Cell Growth Differ. 6*, 409-16.

Yamada, Y., Goto, H. & Ogasawara, N. 1981. Adenosine kinase from human liver. *Biochim. Biophys. Acta 660*, 36-43.

Yamada, Y., Goto, H. & Ogasawara, N. 1982. Deoxyguanosine kinase from human placenta. *Biochem. Biophys. Acta 709*, 265-272.

Yamada, Y., Goto, H. & Ogasawara, N. 1982. Differences of adenosine kinases from various mammalian tissues. *Comp. Biochem. Physiol. [B] 71*, 367-72.

Yoshioka, A., Tanaka, S., Hiraoka, O., Koyama, Y., Hirota, Y. & Wataya, Y. 1987. Deoxyribonucleoside-triphosphate imbalance death: deoxyadenosine-induced dNTP imbalance and DNA double strand breaks in mouse FM3A cells and the mechanism of cell death. *Biochem. Biophys. Res. Commun. 146*, 258-64.

Zullo, S., Sieu, L.C., Slightom, J.L., Hadler, H.I. & Eisenstadt, J.M. 1991. Mitochondrial D-loop sequences are integrated in the rat nuclear genome. *J. Mol. Biol. 221*, 1223-1235.

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